

Development of Broadly Reactive HIV-1/AIDS Virus-like Particle Vaccines

by

Sean Patrick McBurney

B.S. Microbiology, University of Pittsburgh, 2004

Submitted to the Graduate Faculty of
School of Medicine in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

School of Medicine

This dissertation was presented

by

Sean Patrick McBurney

It was defended on

January 27th, 2009

and approved by

Kelly Stefano Cole, Ph.D.

Associate Professor, Department of Immunology

Ronald Montelaro, Ph.D.

Professor, Department of Microbiology and Molecular Genetics

Penelope Morel, M.D.

Professor, Department of Immunology

Karen Norris, Ph.D.

Associate Professor, Department of Immunology

Todd Reinhart, Sc.D.

Professor, Department of Infectious Diseases and Microbiology

Dissertation Advisor:

Ted Ross, Ph.D.

Associate Professor, Department of Microbiology and Molecular Genetics

The vast diversity of HIV-1 infections has greatly impeded the development of a successful HIV-1/AIDS vaccine. Previous vaccine work has demonstrated limited levels of protection against SHIV/SIV infection, but protection was only observed when the challenge virus was directly matched to the vaccine strain. As it is likely impossible to directly match the vaccine strain to all infecting strains in nature, it is necessary to develop an HIV-1 vaccine that can protect against a heterologous viral challenge. Envelope-based vaccines were developed containing gp120 monomers, gp140 trimers and gp160 on the surface of a virus-like particle. Vaccination studies indicated that similar systemic levels of Env-specific antibodies were generated by each vaccine. However the VLP-based vaccine led to increased recognition of Env epitopes as well as a significant increase in mucosal Env antibody responses. Also the VLP-based vaccine led to the development of strong cellular responses to both Env and Gag. Therefore the VLP platform was moved forward to develop broadly reactive vaccines. Consensus and Polyvalent clade B and clade C vaccines were developed and investigated for cellular responses in mice. The results indicated that both Polyvalent and Consensus VLP vaccines led to an increase in the number of cellular Env epitopes recognized as compared with primary Envelope-based vaccines. These findings were true for both clade B and clade C vaccines. To determine the level of protection generated by VLP based vaccines, consensus clade B as well as a polyvalent clade B vaccine were investigated in non-human primates. The polyvalent clade B vaccine led to the protection of 3 out of 4 challenged animals with decreased viral burden observed in the infected individual. Overall these studies indicate that a virus-like particle vaccine encoding multiple primary envelopes is a promising HIV-1/AIDS vaccine strategy for protecting against heterologous HIV-1 viruses.

TABLE OF CONTENTS

1.0	INTRODUCTION.....	1
1.1	FOREWORD	2
1.2	INTRODUCTION	2
1.3	HIV-1 DIVERSITY IN AIDS VACCINE DEVELOPMENT	4
1.4	HIV ENVELOPE.....	7
1.5	CHEMOKINE CO-RECEPTOR USAGE	8
1.6	HIV-1 INFECTION.....	8
1.7	VACCINE APPROACHES TO AIDS.....	10
1.8	CENTRALIZED SEQUENCES.....	12
1.8.1	Centralized HIV-1 Envelope Sequences.	15
1.8.2	Vaccines based upon sequences from a single clade.	16
1.8.3	Vaccines based upon sequences from multiple clades.	17
1.8.4	Vaccines based upon scrambled sequences.	18
1.9	POLYVALENT VACCINES.....	19
1.9.1	Polyvalent Vaccines based upon sequences representing a single protein within a single clade.	20
1.9.2	Polyvalent Vaccines based upon sequences from different clades.	21

1.9.3	Polyvalent Vaccines based upon sequences representing multiple viral proteins.	22
1.9.4	Polyvalent Vaccines based upon Env sequences to increase the breadth of cellular immunity.....	23
1.10	HIV-1 CLINICAL TRIALS.....	24
1.11	CONCLUSION	25
2.0	SPECIFIC AIMS	27
2.1	OVERALL DESIGN AND RATIONALE	27
2.1.1	HIV-1 clade B/R5-tropic vaccine.....	27
2.1.2	Polyvalent vaccines	28
2.1.3	Consensus vaccines	28
2.1.4	Mucosal vaccination of particulate immunogens.....	28
2.1.5	CpG Oligonucleotides.....	29
2.2	OVERALL AIM	30
2.3	SPECIFIC AIM 1	30
2.4	SPECIFIC AIM 2	30
2.5	SPECIFIC AIM 3	31
3.0	SPECIFIC AIM 1.....	32
3.1	FOREWORD	33
3.2	ABSTRACT.....	33
3.3	INTRODUCTION	34
3.4	MATERIAL AND METHODS	37
3.4.1	DNA plasmids.....	37

3.4.2	Transfections and expression analysis	38
3.4.3	Purification of virus-like particles and soluble envelopes	39
3.4.4	Immunization of mice	39
3.4.5	Collection of samples	41
3.4.6	ELISpot assays	41
3.4.7	Antibody response to VLP immunizations	42
3.4.8	Mapping of specific antibodies by poly-L-lysine peptide ELISA	43
3.4.9	Pseudovirus preparation	44
3.4.10	Neutralization assay	45
3.5	RESULTS	45
3.5.1	In vitro expression and particle purification of virus-like particles.....	45
3.5.2	Cell-mediated immunity elicited by VLPs	47
3.5.3	VLP-bound envelope efficiently elicits anti-Env antibody.....	49
3.5.4	Virus-like particles broaden the anti-Env antibody responses.....	51
3.5.5	Neutralization activity	54
3.6	DISCUSSION.....	55
3.7	ACKNOWLEDGMENTS	61
4.0	SPECIFIC AIM 2.....	62
4.1	ABSTRACT.....	63
4.2	INTRODUCTION	64
4.3	MATERIALS AND METHODS.....	66
4.3.1	Envelope gene sequences	66
4.3.2	DNA Plasmids.....	67

4.3.3	Purification of virus-like particles	68
4.3.4	Expression analysis	68
4.3.5	Immunization of mice.	69
4.3.6	Collection of samples.	69
4.3.7	Antibody response to VLP immunizations.	70
4.3.8	Neutralization assay.....	70
4.3.9	ELISpot assays.	71
4.3.10	CD4 cell depletion	72
4.4	RESULTS	72
4.4.1	Selection of wild-type and design of consensus B or C <i>env</i> gene sequences. 72	
4.4.2	Antibodies elicited by VLP vaccines.	79
4.4.3	Elicitation of Cellular Immune Responses.	80
4.4.4	Envelope Cellular Epitopes.....	82
4.5	DISCUSSION.....	89
5.0	SPECIFIC AIM 3.....	94
5.1	FOREWORD	95
5.2	ABSTRACT.....	95
5.3	INTRODUCTION	96
5.4	MATERIAL AND METHODS	98
5.4.1	DNA Plasmids.....	98
5.4.2	Purification of virus-like particles	99
5.4.3	MHC haplotype determination.....	99

5.4.4	Immunization of Rhesus macaques	99
5.4.5	Intravaginal Challenge	100
5.4.6	Collection of Samples.....	100
5.4.7	Viral load Determination	101
5.4.8	CD4/CD8 Cell count	101
5.4.9	CD8 ⁺ lymphocyte depletion.....	102
5.4.10	Antibody Responses to VLP immunization	102
5.4.11	Neutralization Assay	103
5.4.12	ELISpot Assays	103
5.4.13	Antibody-dependent cell mediated virus inhibition.....	104
5.4.14	Statistical analysis	104
5.5	RESULTS	105
5.5.1	Vaccines	105
5.5.2	Immune Responses to VLP vaccination.....	106
5.5.2.1	Breadth of anti-Env antibody responses.....	107
5.5.3	Heterologous vaginal SHIV Challenge.....	111
5.5.4	CD8 ⁺ lymphocyte depletion.....	115
5.5.5	Post-Challenge Immune Responses.....	117
5.6	DISCUSSION.....	120
6.0	SUMMARY AND DISCUSSION	125
7.0	APPENDIX.....	133

LIST OF TABLES

Table 1 Neutralization titers.....	55
Table 2 Characteristics of envelopes used in the virus-like particle vaccines	74
Table 3 Env peptides individually identified by VLP vaccines.....	85
Table 4 Number of peptides identified per region of Env.	87
Table 5 Percent sequence homology with Env peptide sets.	89
Table 6 Virus-like Particle Vaccines	106
Table 7 Individual NHP Coding	106
Table 8 Envelope Sequence Homology	112
Table 9 MHC Alleles	113
Table 10 Pitt Virus-like Particle Vaccines.....	133

LIST OF FIGURES

Figure 1 Genomic organization of the HIV-1 proviral genome.	6
Figure 2 Schematic of HIV-1 envelope.	6
Figure 3 HIV-1 Nomenclature.	7
Figure 4 Centralized Vaccines.	14
Figure 5 Polyvalent Vaccines.	20
Figure 6 Virus-like particle production by sucrose density gradient ultracentrifugation.	47
Figure 7 Elicitation of interferon- γ producing splenocytes.	48
Figure 8 Anti-Env serum antibodies.	50
Figure 9 Total anti-Env IgG and IgA in the mucosa.	51
Figure 10 Location in Env corresponding to high titer induced antibodies recognizing linear epitopes.	53
Figure 11 Generation of consensus (Con B and Con C) envelopes.	79
Figure 12 Envelope expression on virus-like particles.	79
Figure 13 Antibody responses.	80
Figure 14 Anti-envelope cell-mediated immune responses.	82
Figure 15 Identification of envelope-specific peptides.	84
Figure 16 Env regions covered by elicited Env cell-mediated immune responses.	86
Figure 17 Identified epitopes require CD4 ⁺ T lymphocytes.	88

Figure 18 Systemic and mucosal antibody responses are generated by both vaccines.	107
Figure 19 Breadth of pre-challenge anti-Env antibody responses.	110
Figure 20 Anti-SF162 Envelope antibody responses.....	111
Figure 21 PolyB vaccination induced a reduction of viral load following heterologous challenge.	114
Figure 22 PolyB vaccination induced a reduction of viral burden following heterologous challenge.	115
Figure 23 CD8+ lymphocyte depletion leads to expansion of viral load.	116
Figure 24 Viral Infection leads to the expansion of binding and neutralizing antibodies directed to SF162 Envelope.....	118
Figure 25 Post-Infection breadth of anti-Env antibody responses.....	119
Figure 26 Infection leads to the development of anti-SIV Gag cellular Responses.	120
Figure 27 Pre-challenge antibody responses.....	134
Figure 28 Pre-challenge Cellular Immune Responses.....	135
Figure 29 Pitt IR SHIV-SSF162p4 Challenge.....	136
Figure 30 Post-Challenge Anti-Env antibody responses.	137
Figure 31 Post-challenge Cellular Responses.....	138

1.0 INTRODUCTION

Viral sequence diversity: challenges for AIDS vaccine designs

This chapter was modified with permission from:

Sean P. McBurney and Ted M. Ross

Viral sequence diversity: challenges for AIDS vaccine designs

Expert Review of Vaccines November 2008, Vol. 7, No. 9, Pages 1405-1417

© Experts Reviews Ltd

1.1 FOREWORD

Additional information about the immunological immune responses associated with HIV-1 infection and protection as well as further information about HIV-1 envelope was added to the previously published review for completeness. A new discussion of the HIV-1 vaccine trial in Thailand was also added.

1.2 INTRODUCTION

In July, 2008, there were 33.2 million people infected with the human immunodeficiency virus (HIV) with ~2.5 million being new infections [1]. Throughout the world, individuals are infected with a diverse range of HIV isolates [1]. While diversity is an issue with many HIV gene products, the greatest amount of diversity is found in the envelope (Env) glycoprotein. The Env amino acid sequences can differ by as much as 15% between isolates within a single clade and greater than 35% between envelopes from different clades. This diversity is one of the major obstacles facing the development of AIDS vaccines. Therefore, an effective AIDS vaccine must overcome the challenges associated with HIV sequence diversity.

The development of a successful HIV/AIDS vaccine continues to be high-priority for researchers. There have been many failures from the first clinical trials using Env-only based vaccines to the more recent Vaxgen trial (Reviewed in[2-3]). These vaccines failed to induce neutralizing antibody responses. Due to the genetic variability of the viral envelope proteins or the display of epitopes in the proper three-dimensional structure, the virus can escape elicited neutralizing antibodies. In addition, there are difficulties in identifying immunogens and

immunization platforms that consistently induce antibodies that can neutralize isolates from different clades. In light of the difficulties in eliciting neutralizing antibodies, the field switched its focus away from vaccines that induce humoral immunity toward immune responses that reduce viral load and transmission [4-5]. This shift was prompted by data showing that T cell-mediated immunity was critical for resistance to lentiviral infection. However, the recent failure of the candidate vaccine from Merck, which was the first vaccine designed to elicit strong cellular immunity, was a tremendous setback for AIDS vaccine researchers. This Ad5-based vaccine showed no protection against infection and even more significant, the vaccine appeared to increase the rate of HIV infection in individuals with prior immunity against the adenovirus vector. Even though this vaccine elicited HIV-specific immunogenicity in earlier studies, there were limited multifunctional responses.

Therefore, there is a new emphasis towards more basic scientific discovery to overcome the underlying obstacles that hinder the design of an effective prophylactic HIV vaccine. The ultimate goal is to enhance a preventive vaccine that could interrupt HIV transmission and/or substantially control disease progression. This re-focused effort is addressing some fundamental issues associated with HIV infection and transmission to improve the basic understanding of the immune system's response to natural infection and vaccination, to dissect the mechanisms of protection, and to use this knowledge to identify and design effective immunogens and approaches toward manipulating the immune response for an improved vaccine. The use of appropriate animal models for understanding the pathogenesis and transmission of lentiviruses is necessary to construct effective HIV vaccine immunogens. There are many challenges for AIDS vaccine researchers including the development of delivery mechanisms to elicit robust B cell and T cell immunity in appropriate immune

compartments, the use of appropriate animal models to understand the correlates of protection, and vaccine assays to assess the induction of immunity.

1.3 HIV-1 DIVERSITY IN AIDS VACCINE DEVELOPMENT

HIV-1 is a member of the family *Retroviridae* and the genus *Lentiviridae* [6]. The HIV-1 RNA genome encodes the essential retrovirus genes *gag*, *pol*, and *env*, as well as the additional accessory/regulatory genes *vif*, *vpr*, *vpu*, *rev*, *tat*, and *nef* (Fig. 1) (reviewed in [6]). Envelope, located on the surface of viral particles, mediates binding to cellular receptors and entry into cells. The uncleaved envelope protein (Env_{gp160}) is a highly glycosylated molecule that helps mask it from the immune system. Env_{gp160} is cleaved into Env_{gp120} and Env_{gp41} [7]. On the surface of the virion, envelope forms trimers with Env_{gp120} on the outer surface bound to Env_{gp41} spanning the cellular membrane [8]. Env_{gp120} is composed of the constant regions C1-C5 and the variable regions V1 to V5 (Fig. 2).

A central feature of lentiviruses in general, and HIV-1 in particular, is the propensity for these viruses to rapidly mutate and evolve in response to immunological or other selective pressures. While all of the viral genes are susceptible to mutation and protein variation, the envelope proteins are hotspots of sequence variation, as they are primary targets for host immune responses, and Env has evolved to permit substantial sequence variability to confound immune recognition and control. Thus, HIV-1 populations at the individual and global levels exist as a heterogeneous mixture of genomic quasispecies unique to a patient or on a larger scale to a geographical area. With all of this diversity, it is reasonable to ask whether there is any commonality that can be identified and targeted for vaccine development among diverse HIV-1

envelope species. Based on viral genetic distances and positions in phylogenetic trees, HIV-1 has been divided into three groups: M, O, and N (Fig. 3). Group M viruses are found globally and largely responsible for the AIDS pandemic, while groups O and N viruses are restricted to west central Africa. Viral envelope sequences differ by up to 50% between these groups. Within group M, HIV-1 isolates have been further divided into distinct clades (designated A-K) that differ in envelope sequence by as much as 35% [9]. Envelope sequences can also differ by up to 15% between viruses in the same clade. There are also circulating recombinant forms (CRFs). CRFs are viral isolates that are the combination of two or more viral isolates from different clades that have been found in at least three epidemiologically independent individuals [10]. The combination of the high diversity and isolates within clades and between clades indicates that a vaccine based on a single isolate is unlikely to provide protection against all possible exposures to HIV-1 isolates. This point is further emphasized by studies that show protection can be elicited by vaccines against homologous challenge viruses, but not against heterologous challenges [11-13].

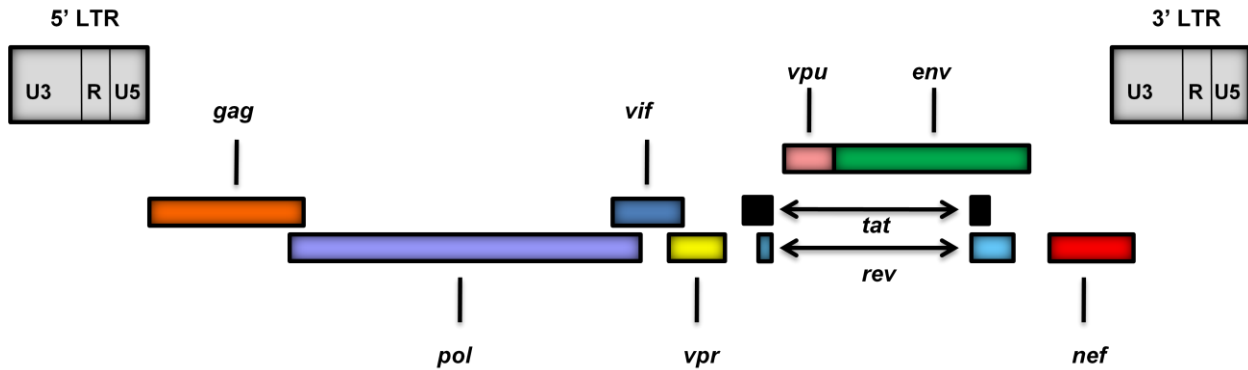


Figure 1 Genomic organization of the HIV-1 proviral genome.

Structural and enzymatic proteins are encoded by the *gag*, *pol*, and *env* genes. Regulatory gene products are encoded by the *tat* and *rev* genes and the major accessory proteins are encoded by the *vif*, *vpu*, *vpr*, and *nef*. The long terminal repeats (LTR) are sites for initiation of viral RNA synthesis and necessary for proviral integration into host cell chromosomes.

A.



B.

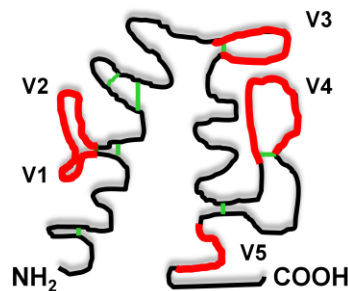


Figure 2 Schematic of HIV-1 envelope.

(A.) shows the division of Env_{gp160} into gp120 and gp41 as well as the regions of Envelope and their location within gp120 and gp41. (B.) A Schematic of folded gp120. The constant regions are shown in black with the variable regions shown in red. Disulfide bonds are shown in green. Structure was determined by Leonard et al[14].

HIV-1

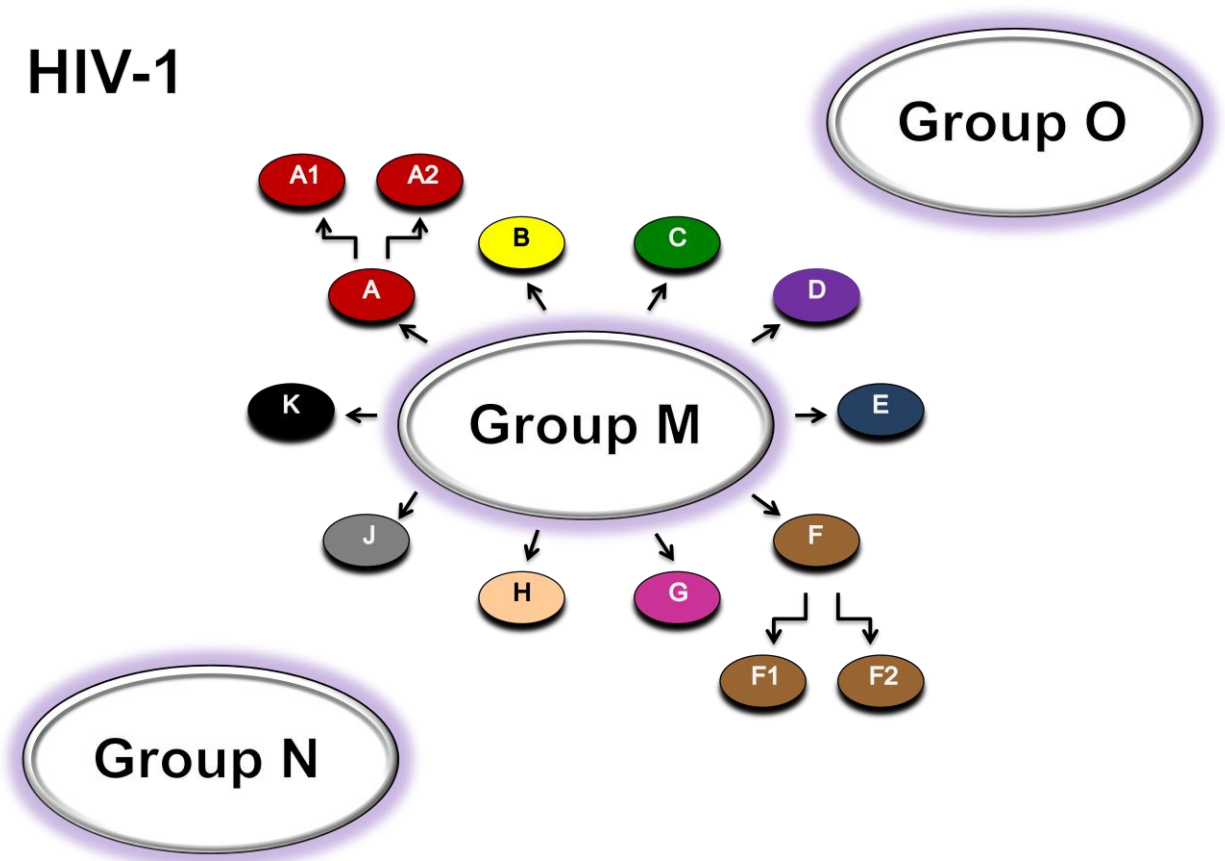


Figure 3 HIV-1 Nomenclature.

HIV-1 is divided into three Groups: Main (M), Outlier (O) and Non-M/Non-O (N). Groups M, O, and N are shown as large ovals. Group M is further divided into clades which are shown as small ovals arrows pointing from the Group M oval. Clades A and F have also been further divided into A1/A2 and F1/F2.

1.4 HIV ENVELOPE

Envelope is necessary for receptor binding and viral entry [15-18]. The *env* gene is expressed as a polyprotein precursor, Env_{gp160}. Env_{gp160} is then cleaved by a cellular protease, furin, into the two Env glycoproteins, gp120 and gp41 which associate through noncovalent

interaction. These molecules form a multimeric structure (most likely a trimer) on the surface of the virion [19-22]. Env_{gp41} forms the transmembrane domain of the Env complex, while Env_{gp120} is presented on the surface of infected cells or virions. Upon infection, Env_{gp120} binds to human CD4 (hCD4) on the surface of target cells [23-26]. This interaction results in a conformational change in Env_{gp120}, which exposes the chemokine receptor-binding domain. Binding of the virus to the chemokine receptor (CCR5 or CXCR4) leads to another conformational change in Env_{gp120} that exposes the fusogenic domain in Env_{gp41}. The fusogenic domain instigates entry of the viral core by fusing the viral and host cell membranes [23, 27-28].

1.5 CHEMOKINE CO-RECEPTOR USAGE

The binding of the chemokine co-receptor is a necessary step in viral infection as stated above. However, different HIV-1 isolates utilize different chemokine receptors for viral entry. Sexually transmitted viruses and virus isolated early after infection primarily utilize the CCR5 receptor[29]. As the disease progresses, there is an expansion in receptor utilization. Viral isolates found years after the initial infection or during late-stage disease, predominately initiate infection through the binding to CXCR4[29].

1.6 HIV-1 INFECTION

There are four major modes of HIV-1 transmission: 1) sexual intercourse, 2) blood products, 3) contaminated needles and 4) mother-to-child during the prenatal period

[30]. After infection the time until disease progression can vary greatly between individuals. In general, the first symptoms of clinical AIDS become evident 8-15 years after infection.

HIV Infection has three distinct phases. The first phase is an acute infection which is marked by rapid replication of virus followed by a steady decline in virus levels. This phase is often accompanied by flu-like symptoms which go unrecognized as being caused by HIV-1 infection. During this phase the activation of cytotoxic T cells (CTL) and induction of anti-HIV antibodies result in containment of the initial virus. An increase in CD8⁺ cytotoxic T lymphocytes is also often seen, but neutralizing antibodies are not detected until 6 months after infection. CD4⁺ T lymphocytes decrease during this phase but return to near normal levels after six months.

The second phase of HIV-1 is characterized by low levels of detectable virus and maintained CD4 counts. The level of detectable virus (set-point) is often prognostic of the disease course. High set-points are associated with rapid disease progression while low set-points to undetectable virus are associated with slow to non-progression of disease [31-33]. During this phase most infected individuals remain asymptomatic.

The final phase of disease is characterized by a sharp increase in viral load as well as decreasing CD4 lymphocyte levels and is most associated with AIDS. AIDS is characterized by a state of immunodeficiency that allows for the development of secondary, opportunistic infections. Some of the most prevalent opportunistic infections are *Pneumocystis carinii*, *Cryptosporidium*, *Toxoplasma*, *Mycobacterium*

avium/tuberculosis and *Salmonella* [34-39]. Tumors are also usually found in AIDS patients some of the most common are Kaposi's sarcoma, non-Hodgkin's lymphoma and primary lymphoma of the brain [40-43].

1.7 VACCINE APPROACHES TO AIDS.

The ultimate goal of an AIDS vaccine is to elicit potent cellular and humoral immune responses that will result in enduring, broadly protective immunity. While much effort has focused on elucidating the mechanisms and specificity of cellular immune responses, less is known about virus-specific antibody responses. It has been well established that cellular immune responses mediate viral control during the primary infection and antibody depletion studies in the SIV/monkey model also demonstrated a role for cellular immunity in maintaining the viral set-point in chronic infection [44]. On the other hand, passive protection experiments using human monoclonal antibodies resulted in protection against virus exposure in the HIV-1/chimp [45-46], SIV/monkey [47-50] and SHIV/monkey models [51-57], demonstrating the ability of antibody alone to mediate protection against pathogenic virus infection. Thus, the goal of an effective AIDS vaccine should be to elicit as robust and broadly reactive cellular and humoral immunity as possible, thereby maximizing the potential for protection from variant HIV-1 strains by different routes of exposure.

Initial trials using monomeric envelope proteins elicited high titer antibody responses against the immunogen [58-65]. However, while these single envelope vaccines have proven protective in both SIV and HIV non-human primate challenge models, protection was only observed when the envelope in the vaccine was precisely matched to the envelope on the

challenge virus [11-13]. These results suggest that envelope diversity is an important obstacle in the development of an effective AIDS vaccine. This is supported by the fact that single envelope vaccines have not been successful in humans, most likely due to the inability to pre-select the challenge virus in humans and the tremendous diversity of HIV-1 isolates.

Recent vaccine studies using a polyvalent Env vaccine elicited broader immune responses, particularly neutralizing antibodies, but did not provide sterilizing protection against heterologous SHIV infection in pigtailed macaques [66]. Interestingly, animals in the multivalent vaccine group, but not those immunized with the monovalent vaccine, exhibited markedly lower levels of plasma virus than monkeys in the control group, suggesting superior cell-mediated responses induced by the polyvalent vaccine [66]. Therefore, it appears that immune responses capable of recognizing conserved epitopes within Env, with the concomitant ability to recognize these epitopes on more than one strain of HIV-1, may play an important role in inhibiting the viral spread *in vivo*. In addition to Env stimulating classical cellular and neutralizing antibody responses, due to the surface exposure of Env on both virions and infected cells, antibodies can also support complement-mediated lysis of virions and antibody dependant cellular cytotoxicity (ADCC) of infected cells. Despite a lack of reliable correlates for immune protection against HIV-1 infection, an effective vaccine against HIV or AIDS will most likely need to elicit high levels of cross-reactive neutralizing antibodies in combination with a robust cell-mediated response against multiple viral antigens. Toward this end, researchers have demonstrated a critical correlation of the maturation of immune responses to SIV, SHIV, and other lentivirus envelope proteins and the development of protective immunity in animals inoculated with live attenuated vaccine strains [50, 67-73]. Thus, an effective AIDS vaccine

may need to include a full-length, native envelope protein to enhance the breadth and maturation of both cellular and humoral immune responses.

Neutralizing antibodies, CD4⁺ and CD8⁺ T-cell responses, and innate immunity have all been linked to protective efficacy. However, none of these immune parameters are universal. Vaccine studies performed in non-human primates (NHP) indicate that protection from infection may be possible in humans [74-77]. Passively transferred neutralizing antibodies can protect against infection, but neutralizing antibodies have been difficult to elicit by vaccination. Live-attenuated SIV vaccines are able to protect NHP against subsequent infection [78-80]. Due to the possibility of reversion, live-attenuated HIV-1 vaccines are not suitable for human use. However these types of vaccines do offer an effective mechanism for studying correlates of protection. Results from live-attenuated SIV infection studies indicate that cellular and humoral immunity elicited at the site of challenge can offer some level of protection from disease [78-80]. However, these responses were only protective when the challenge virus was homologous to the vaccine. These monkeys were not protected against heterologous challenge viruses indicating that these vaccines elicited a restricted breadth of immunity [66, 81-82]. Due to the vast diversity of HIV-1 isolates circulating in the population, it is unlikely that a vaccine based on a single viral isolate will be able to protect against human infection.

1.8 CENTRALIZED SEQUENCES

Centralized sequences are designed to limit the genetic distance between any given viral isolate and the vaccine strain. Vaccines using this approach can be designed against any protein. Three primary methods have been used to design centralized vaccines for HIV (Fig. 4). Each method

utilizes a given population of sequences that are based on a diverse set of parameters, such as the regional location of viral isolation, the HIV clade, the year of isolation, or the specific co-receptor usage. A phylogenetic tree is then developed using primary viral protein sequences to develop one of two types of centralized sequences: center-of-the-tree or ancestral. Center-of-the-tree sequences are based upon generating a sequence that is equidistant to all points of the tree [9, 83]. The ancestral approach is based upon the theoretical ancestor sequence that gave rise to all sequences of a particular tree [9, 83]. In addition, the population of these sequences can also be aligned to make a consensus sequence by assigning the most common amino acid located at each position [9, 83]. Generation of hybrid consensus sequences of the Env_{gp120} constant regions have been used in conjunction with the variable regions based upon a single viral isolate [84]. HIV vaccines based upon each of these centralized methods are in development.

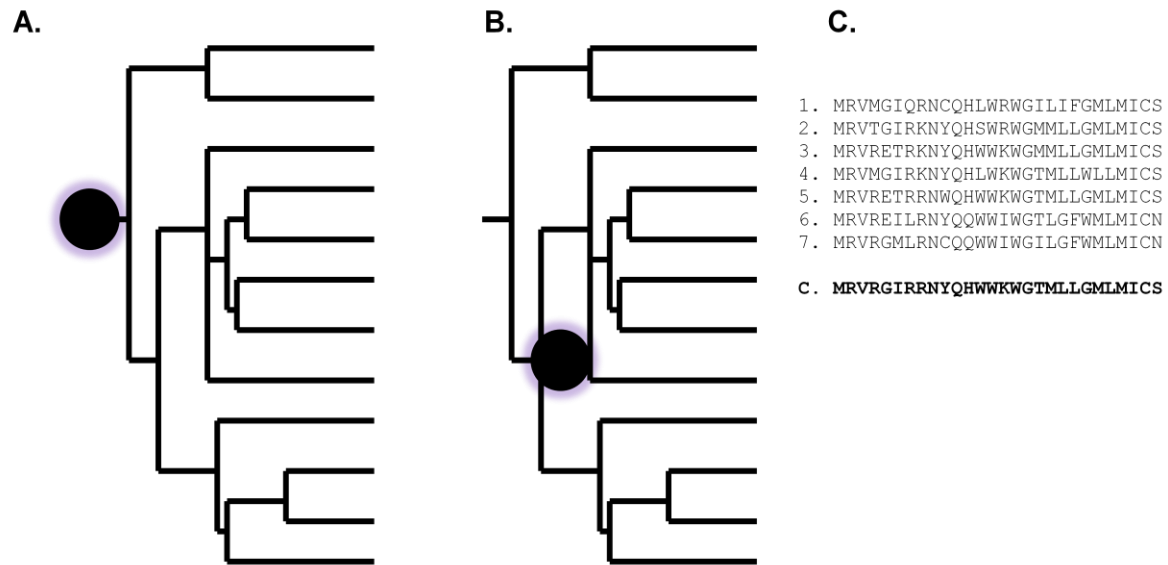


Figure 4 Centralized Vaccines.

This figure demonstrates the three methods of developing a centralized vaccine. (A.) Ancestral vaccine. This method of centralization utilizes the theoretical ancestor that gave rise to the phylogenetic tree. The location of the ancestral vaccine is shown with a black dot. (B.) Center-of-the-tree. This method of centralization generates a sequence that is equidistant to all points of the phylogenetic tree. The approximate location of the vaccine sequence is shown with a black dot. (C.) Consensus vaccine. A consensus sequence was generated from the seven sample envelope sequences by assigning the most common amino acid at each position in the amino acid sequence.

The development of centralized sequences is highly dependent on the quality of the starting sequence library. The center-of-the-tree and ancestral methods utilize a phylogenetic tree that is constructed based on assumptions created to fill gaps generated by incomplete sampling of the viral population. If these assumptions are incorrect it will alter the resulting centralized sequence to also be incorrect. The greatest degree of problems comes with the development of ancestral sequences, as they are the most heavily influenced by the assumptions used to generate

a phylogenic tree. Sampling for the development of ancestral sequences also require including sequences from older samples. These samples are often difficult to obtain and are representative of only a small population of viruses thus affecting the resulting sequence. A consensus sequence can also be biased by the sampling of the viral population. If this sampling only covers a small population, the resulting sequence will be unevenly weighted to that sample. Each centralized strategy is dependent on developing an unbiased, large database of sequences. Through the development of such databases the short comings of these methods can be overcome.

1.8.1 Centralized HIV-1 Envelope Sequences.

The three methods of developing centralized sequences are currently being used to overcome the challenges of viral diversity in HIV-1. The most common approach has been through the use of consensus sequences. However, any of the three designs may miss epitopes in any given immunogen therefore combining several consensus sequences has advantages for covering as many epitopes within a diverse immunogen, such as the HIV-1 envelope. Recently, vaccine strategies utilizing consensus and ancestral Env epitopes have proven potent inducers of both humoral and cellular immunity [85-89]. The initial generation of centralized sequences were tested to ensure that these artificial genes were able to express functional products [85, 90-94]. The majority of centralized sequences use codon optimization to enhance gene expression. This technique generates a sequence using the optimum codon for a given species to maximize protein expression [95]. Codon optimized envelopes are cleaved into Env_{gp120} and Env_{gp41} at the surface, similar to native Env proteins [91-92]. Centralized envelopes form trimers that facilitate binding

to CD4 and co-receptors, as well as establish infection *in vitro* when pseudotyped onto viral backbones [85, 91-92, 94]. Other consensus HIV-1 proteins such as Tat, Rev and Nef have also been shown to efficiently express and be naturally processed further demonstrating the utility of consensus sequences [93].

Centralized Env vaccines based upon geographic regions, specific Group M clades, multiple clades and the entire Group M have been investigated [84-85, 90-94, 96-104]. Regional centralized vaccines based upon viruses circulating in specific geographical regions of Africa [99, 103] were designed to match the viral strains which caused over 70% of the infections within an area, such as Kenya [99]. This vaccine consisted of a consensus CA_{p24}/MA_{p17}, as well as CTL epitopes derived from a clade A Env. Strong cellular immune responses were observed in murine studies and immunogenicity was demonstrated in early clinical trials. A vaccine designed for eastern and central Africa consisted of a consensus clade A Tat, Reverse Transcriptase, Nef and Env_{gp41} [103]. This vaccine induced strong cellular responses to each of the above components; however it did not elicit antibodies, which may limit its effectiveness in protective efficacy.

1.8.2 Vaccines based upon sequences from a single clade.

Centralized vaccines targeted against clade B or C gene products elicit strong cellular and humoral immune responses [91-93]. A DNA vaccine based upon centralized clade C sequences expressing Tat, Rev and Nef elicited cellular immune responses that were equal to immunity elicited by corresponding vaccines based upon wild-type, primary sequences [93]. In addition, ancestral and consensus envelope-based vaccines for clade C are highly immunogenic eliciting both strong cellular and humoral immune responses [92]. These centralized Env-based vaccines

elicited antibodies that recognized an increased number of clade C isolates compared to vaccines composed of wild-type sequences. Consensus clade B Envs elicit an increased breadth of antibody recognition and virus neutralization as compared to primary isolates[91]. Interestingly, similar cellular responses were elicited by both clade B and C vaccine approaches [92]. These results indicate that vaccines based upon a centralized sequence can increase the breadth of immune responses, thereby allowing for an increase in the coverage of circulating viruses.

1.8.3 Vaccines based upon sequences from multiple clades.

Centralized vaccines based upon the entire Group M set of isolates strive to elicit immunity against various proteins in viral isolates across clades [84-85, 94, 102]. A group M consensus Env vaccine induced both cellular and humoral immune responses [84-85, 94]. These vaccines induce immune responses that recognize both clade B and C cellular epitopes, as well as antibodies that neutralize isolates in both clades. Compared to vaccines using Envs derived from wild-type clade A, B, and C isolates, the consensus M Env vaccine elicited higher titer immune responses within each respective clade than vaccines based upon the wild-type sequences [84]. These results indicate that a group M consensus vaccine can generate both high titer and broader immune responses that may elicit protection against current and emerging HIV isolates.

In contrast to a single consensus M Env strategy, a vaccine based upon Env sequences (consensus or ancestral) from multiple clades (A, B, C, F, G, and H) is currently in pre-clinical testing [102]. This DNA vaccine is composed of *rev*, *nef*, *tat*, and *gag* consensus sequences derived from clades A, B, and C and an ancestral sequence derived from clades F, G, and H [102]. This vaccine was given along with a second vaccine which encoded known cellular Env

and Pol epitopes [102]. In mice, this vaccine strategy elicited strong cellular responses to Gag and Env that protected against murine leukemia virus (MuLV) with envelopes derived from clade A and clade B viruses. Overall, a multiclade centralized vaccine strategy elicits immune responses that recognize multiple HIV proteins from multiple clades. These vaccines demonstrate that group, clade, and regional centralized vaccines are immunogenic and that each type of vaccine is able to elicit increased coverage of viral isolates compared to wild-type based vaccines.

1.8.4 Vaccines based upon scrambled sequences.

Consensus methods are also being used to deliver whole genome vaccines. The synthetic scrambled antigen vaccine (SAVINE) is based on a weighted consensus genome of clades A, B, C, and E [104]. The SAVINE vaccine is a novel use of centralized sequences. The vaccine is divided into three expression plasmids expressing scrambled peptides encompassing all the HIV gene products. SAVINE was designed using a consensus genome sequence. The HIV proteins were then divided into 30-mer peptides overlapping by 15 amino acids. These peptides were expressed as a single polypeptide from sequences that were randomly joined into a single cDNA. Due to the size of this cDNA, it was divided into three separate open reading frames (ORF). Each of the ORFs contained multiple small sections of various HIV proteins, thereby resulting in a single protein that presents multiple epitopes from every protein of the virus. The SAVINE vaccine is highly recognized by HIV-1 positive patient sera demonstrating that the SAVINE vaccine presents epitopes, even though it does not encode any natural forms of HIV proteins[104]. The SAVINE vaccine is highly immunogenic inducing strong cellular responses

in mice [104]. The advantage of the SAVINE vaccine is it can present epitopes from all of the HIV gene products without expressing a complete HIV protein.

1.9 POLYVALENT VACCINES

An alternative strategy to centralized sequences is the use of mixtures of the same immunogen from different isolates. These polyvalent vaccines aim to increase the coverage of the vaccine by combining multiple components into a single vaccine. This strategy has been used successfully against infectious disease pathogens, such as pneumococcus, poliovirus and influenza virus [105]. Multiple methods for making a polyvalent HIV vaccine have been used (Fig 5). One method of making a polyvalent vaccine is to include multiple components of the virus together in the vaccine. These methods are not designed to increase the coverage of viral isolates, but are instead to increase the breadth of the immune responses against a single isolate by eliciting immune responses to multiple components. The second method is to use antigens from multiple viral isolates. These vaccines can be developed using either single antigens or multiple antigens from multiple isolates. In this way a single vaccine can employ both methods of making a polyvalent vaccine.

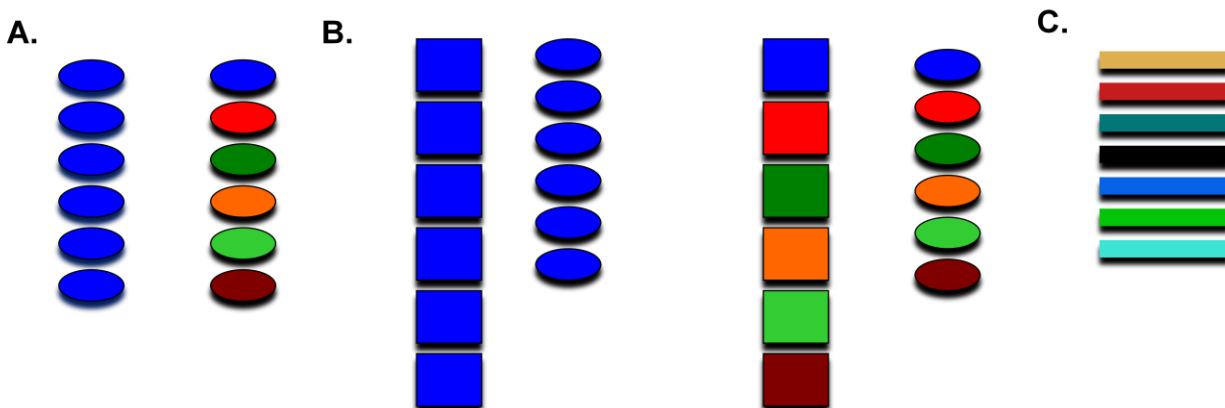


Figure 5 Polyvalent Vaccines.

This figure demonstrates the three main methods of developing a polyvalent vaccine. (A.) Single component vaccine. These polyvalent vaccines are composed of a single repeated component shown as an oval. These vaccines can be based on a single clade shown in blue or multiple clades as demonstrated by multiple colors. (B.) Multiple component vaccine. These polyvalent vaccines are composed of two or more target proteins and can also be based on single or multiple clades. One antigen is shown as a square with the second antigen being shown as an oval. (C.) Polyvalent Peptide vaccines. These vaccines are designed to present the most common cellular epitopes from a population of sequences to the immune system. The different colors represent different epitopes.

1.9.1 Polyvalent Vaccines based upon sequences representing a single protein within a single clade.

The simplest polyvalent vaccines are those consisting of multiple repeats of a single protein. The Env immunogen has been the main focus of this type of polyvalent vaccine due to its high sequence diversity using envelopes from a single clade or from multiple clades. Polyvalent vaccines containing either four or five clade B envelopes protected vaccinated monkeys against SHIV_{DH12} infection and neutralized a greater number of viral isolates than the monovalent vaccines, but this increased recognition was primarily restricted to the viral isolates included in

the vaccines [66]. These results indicate that, while a polyvalent vaccine can increase the breadth of a vaccine, this breadth may be limited. Both the monovalent and polyvalent vaccine groups containing the challenge strain elicited greater protection than either the monovalent or polyvalent vaccine, which did not contain the SHIV_{DH12} Env. These results indicate a direct link of neutralizing antibody titers to protection against SHIV_{DH12} infection [66]. In addition to neutralizing antibody, other immune components contributed to protection. Monkeys vaccinated with the polyvalent vaccine without the SHIV_{DH12} Env had lower plasma viremia than monkeys vaccinated with the matched monovalent vaccine [66]. These results, along with other studies [106], indicate that polyvalent Env vaccines elicit broader cellular immune responses that partially control viral infection. Even a polyvalent vaccine composed of 14 Envs induced stronger cellular and humoral responses than a monovalent vaccine [106]. Interestingly, even though this vaccine was composed of exclusively clade B envelopes, it generated cellular immune responses which recognized epitopes from clades C and A/E viruses [106]. Therefore, a polyvalent vaccine composed of Envs from a single clade can induce broader humoral and cellular immune responses than a monovalent vaccine, and these responses may also recognize viral isolates or epitopes from different clades.

1.9.2 Polyvalent Vaccines based upon sequences from different clades.

To address the worldwide diversity of HIV, polyvalent vaccines composed of envelopes from different clades are in pre-clinical testing. A majority of these studies use polyvalent vaccines composed of isolates from clades A, B, and C [107-109] and one study used isolates from clades A, B, C, D, and E [110]. These multiclade polyvalent vaccines induce broader neutralizing

antibodies than monovalent vaccines against each clade [107-110]. The multiclade vaccines also increase the breadth of cellular immunity compared to a monovalent vaccine [108-109]. Envelope only vaccines using either multiclade or single-clade polyvalent strategies increase the breadth of both cellular and humoral immunity against Env. This approach may be feasible for other HIV antigens or immunogens from other viruses with a high degree of diversity.

1.9.3 Polyvalent Vaccines based upon sequences representing multiple viral proteins.

To target a wider range of HIV proteins, polyvalent vaccines incorporating multiple Env isolates as well as other viral proteins have been constructed to address the Env sequence diversity while combining these immunogens with other viral targets. These vaccines increase the breadth of cellular and humoral immune responses to envelope similar to the increase observed with Env only polyvalent vaccines [111-119]. In addition, these vaccines elicited robust immune responses against Gag epitopes [111-119] and several of these vaccines protected monkeys against SHIV infections [115-116]. One of the limitations of these challenge studies was the use of a challenge strain that was homologous to one of the Env isolates within the polyvalent vaccine [115-116], therefore, it is unclear if these vaccines will protect against a truly heterologous virus. A recent clinical phase 1 trial using a polyvalent vaccine incorporating Env from clades A, B, C, and E, as well as a Gag p24 from clade C induced strong cellular immune responses against A, B, C, and E envelopes, as well as Gag epitopes [119]. Antibody binding and neutralization were observed against a wide range of viral isolates from clades A, B, C, D, and E [119]. These encouraging results indicate that a polyvalent HIV-1 vaccine is a viable vaccine strategy for humans.

1.9.4 Polyvalent Vaccines based upon Env sequences to increase the breadth of cellular immunity.

A modified polyvalent vaccine, designed for optimum coverage of potential T-cell epitopes, utilizes a large population of sequences similar to those used in centralized sequence design [120]. These populations are used to generate mosaic sequences by using recombination events that are designed to generate a sequence, which provides optimum coverage of the most common 9-mer epitopes for a given target protein. These mosaic sequences exclude any rare 9-mer epitopes, so only the most likely epitopes are included in the final product. These computationally-derived mosaic vaccines were compared to both polyvalent vaccines using natural strains and consensus vaccines. The computational data indicates that the optimized polyvalent mosaics will elicit broader coverage of potential T-cell epitopes than either natural polyvalent or consensus vaccines [120], but these assumptions still need to be tested in animal models to determine if the computational increase in T-cell epitope coverage will result in increased protection or immunological recognition. This study does demonstrate a new method of designing future vaccines through the use of computational methods.

Another modified polyvalent strategy designed to target multiple clades utilizes sequences from 21 clade A, 128 clade B, 51 clade C, 17 clade D, and 33 clade E isolates to generate 96 lipidated and 80 non-lipidated peptides [121]. These peptides represent the five hypervariable regions of the HIV-1 gp120 and the two variable epitopes in HIV-1 Gag [121]. The peptides were combined and delivered subcutaneously to *Cynomolgus macaques* and humanized HLA-A2.1 mice. The vaccine elicited strong CD4⁺ and CD8⁺ T-cell responses that

were directed to isolates from clades A-E demonstrating that a multiclade polyvalent vaccine can elicit cellular immune responses to viruses from each of the clades included in the vaccine. Even though this vaccine was designed to elicit mostly cellular immune responses, moderate humoral responses were also observed. Low levels of binding antibodies were observed against the vaccine peptides, as well as primary Env isolates from clades A-E. Moderate neutralization was observed against T-cell line adapted virus with weak neutralization observed against a few primary isolates [121]. Viral challenges were completed in humanized mice against a recombinant vaccinia virus expressing HIV-1_{IIIB} Gag/Pol/Env proteins. Vaccinated mice had a 2-3 fold reduction in viral titer demonstrating control and clearance of infection [121]. It is unclear if this challenge model can be correlated to non-human primate challenge systems. Together, all of polyvalent vaccines demonstrate that there is not a loss of immunogenicity when additional HIV targets are added to the vaccine. This is important as it is unlikely that a vaccine which focuses on only one target of HIV will be successful in the long term due to the high mutation rates of HIV.

1.10 HIV-1 CLINICAL TRIALS

The recent apparent failure of the Merck HIV-1 vaccine trial has led many to question our ability to develop an effective vaccine. The results of these trials have been extensively reviewed elsewhere and will only be mentioned briefly as they apply to the development of broadly reactive vaccines (see reviews [122-123]). The vaccines tested in these trials were designed using single isolates. They therefore were only able to generate limited reactivity against heterologous viruses. These vaccines were able to protect against homologous infections in non-

human primates, but proved to be ineffective against human infections. This is not because the vaccines are not inducing strong immune responses in humans, but is likely due to the fact that the infecting viruses are no longer homologous to the vaccine. The recent successful Thai study was based on three separate Env isolates as well as Gag and Pol antigens[124]. This formulation likely led to the development of a more broadly reactive immune response. This vaccine was focused on the development of antibody responses to the different Env components indicating the possibility of an effective antibody based vaccine. Initial results from this study do indicate that the vaccine did not have any effect on post-infection viral levels or immune responses. This may be due to the fact that antibody responses will mostly work at blocking the initial infection of the virus, but if an infection is established the antibody responses are not sufficient to clear the virus. Therefore further work is needed to continue the development of these broadly reactive vaccine strategies and to bring these strategies forward into clinical trials.

1.11 CONCLUSION

Any future HIV-1 vaccine should be broadly reactive. This study has chosen to focus on the development of an Env-based HIV-1 vaccine. The reason that envelope was chosen was because it has been previously shown that antibody responses to Env if present in sufficient quantities are capable of blocking HIV-1 infection. These antibody responses are capable of sterilizing immunity such that virus infection is never established and the exposed individual remains free of residual virus. The first step in this work was to determine the best method for the presentation of Env as an immunogen. Once the best presentation, method was identified two methods of developing broadly reactive immune responses were compared to determine which

method induces the broadest range of Env recognition. The consensus method was selected from the centralized vaccine techniques as this technique is least limited by computational design and is better able to resemble a contemporary early viral isolate than either center-of-the tree or ancestral methods. The polyvalent method was selected as it is a well established technique that has been used in multiple vaccines against other diseases. Only early Env isolates were included within the polyvalent method as these isolates are more similar to transmitted viruses than late stage isolates. The consensus and polyvalent vaccines were also tested for their ability to protect against a heterologous viral challenge as it is currently unclear as to what degree of breadth is needed to facilitate protection against heterologous viral strains.

2.0 SPECIFIC AIMS

2.1 OVERALL DESIGN AND RATIONALE

The overall aim of this project was to develop a broadly reactive HIV-1/AIDS vaccine. The vast diversity of HIV-1 makes it highly unlikely that a single primary isolate will be able to induce immune responses that will be cross-reactive against the breadth of possible viral isolates. The focus of these studies is the development of an envelope-based vaccine that will be able to induce the greatest breadth of humoral and cellular immune responses.

2.1.1 HIV-1 clade B/R5-tropic vaccine

The majority of individuals in the United States and Western Europe are infected with HIV-1 clade B strains. Viral transmission is almost always completed by R5-tropic isolates. For this reason the vaccines were developed to express clade B, R5 isolates that were isolated soon after infection. It is thought that these isolates will be better able to mimic transmitting viruses thereby generating greater protection. Clade C viral isolates were also investigated to determine the cross-clade reactivity of the vaccines from different clades. If cross-clade protection is not observed it may be necessary to develop multiple clade specific vaccines to best protect individuals from the range of HIV-1 worldwide.

2.1.2 Polyvalent vaccines

Polyvalent vaccines in other systems have been shown to increase the breadth of the immune responses compared to single isolate vaccines. The use of multiple isolates allows for the generation of immune responses to epitopes common to all isolates found in the vaccine as well as to epitopes found in only a single isolate. This generates immune responses that are able to recognize a greater number of epitopes thereby increasing the likelihood of being able to recognize an epitope or epitopes from an infecting isolate.

2.1.3 Consensus vaccines

Consensus vaccines are generated by developing an artificial sequence that matches the most common amino acids at each position in the antigen of interest. It is thought that this will technique will be able to generate an immune response that recognizes epitopes found in most isolates that would be encountered. Whereas a single isolate would generate immune responses to epitopes that may be unique, a consensus vaccine would generate immune response to epitopes common to most isolates. This would facilitate protection against a greater breadth of isolates than a given single isolate.

2.1.4 Mucosal vaccination of particulate immunogens

HIV-1 is primarily transmitted through the mucosal route. A protective vaccine must induce a mucosal immune response to block infection at the site of entry. Mucosal immunization can induce immune responses at mucosal sites as well as systemically, but systemic immunization is

often unable to induce responses at mucosal sites. Therefore, a mucosally delivered HIV-1/AIDS vaccine would be best able to induce protection if delivered through a mucosal route.

The mucosal route chosen for this study was nasal administration. Vaccines delivered nasally can be phagocytosed by M cells in the nasal lumen and then directly deposited to the NALT via M cell transcytosis, which preferentially drains into cervical lymph nodes. This process generates a strong immune response in the respiratory track. More importantly for HIV-1 transmission, it is also able to induce strong immune responses in the genital mucosa at which a majority of HIV-1 infections occur. Systemic immune responses are also established providing protection if the infection is not stopped at the mucosal surface.

2.1.5 CpG Oligonucleotides

Synthetic oligodeoxynucleotides contain unmethylated CpG motifs that are recognized by Toll-like receptor 9. CpGs induce an inflammatory state in dendritic cells and B cells increasing antigen uptake and processing thereby facilitating a stronger immune response. CpGs have also been shown to work as a strong adjuvant when given via the nasal route.

2.2 OVERALL AIM

To determine the best strategy for development of a broadly reactive and protective Env-based HIV-1/AIDS vaccine.

Hypothesis: A virus-like particle based- Consensus Envelope vaccine will generate the greatest breadth of humoral and cellular immunity facilitating protection against heterologous viral challenge.

2.3 SPECIFIC AIM 1

To determine if soluble forms of Env elicit similar immune responses as native, membrane bound Env.

Hypothesis: Envelope expressed on the surface of a virus-like particle elicits broader humoral and stronger cellular immune responses than soluble gp120 monomers and soluble gp140 trimers.

2.4 SPECIFIC AIM 2

To compare the breadth of cellular immunity elicited by consensus HIV-1 VLP and Polyvalent HIV-1 VLP vaccines.

Hypothesis: A Consensus VLP vaccine will elicit a broader cellular immune response than a polyvalent VLP.

2.5 SPECIFIC AIM 3

To compare the breadth of humoral immunity elicited by consensus HIV-1 VLP and Polyvalent HIV-1 VLP vaccines and to determine the protective effect of these vaccines against a heterologous viral challenge.

Hypothesis: Polyvalent and Consensus vaccines will provide protection against an intravaginal SHIV_{SF162p4} challenge with the Consensus B VLP vaccine eliciting the greatest breadth of immunity.

3.0 SPECIFIC AIM 1

**Membrane embedded HIV-1 envelope on the surface of a virus-like particle elicits broader
immune responses than soluble envelopes**

This chapter was modified with permission from:

Sean P. McBurney, Kelly R. Young, and Ted M. Ross

Membrane embedded HIV-1 envelope on the surface of a virus-like particle elicits broader
immune responses than soluble envelopes

Virology. 2007 Feb 20;358(2):334-46

Copyright © 2006 Elsevier Inc.

3.1 FOREWORD

The work described in this aim was completed with the assistance of Kelly Young. Dr. Kelly Young completed the first set of mice done for this study, as well as evaluating the initial gp120 binding and cellular immune responses from this group. Two additional groups, as well as all other immunological testing, were completed by Sean P. McBurney.

3.2 ABSTRACT

Virally regulated HIV-1 particles were expressed from DNA plasmids encoding Gag, protease, reverse transcriptase, Vpu, Tat, Rev, and Env. The sequences for integrase, Vpr, Vif, Nef, and the long terminal repeats (LTRs) were deleted. Mutations were engineered into the VLP genome to produce particles deficient in activities associated with viral reverse transcriptase, RNase H, and RNA packaging. Each plasmid efficiently secreted particles from primate cells in vitro and particles were purified from the supernatants and used as immunogens. Mice (BALB/c) were vaccinated intranasally (day 1 and weeks 3 and 6) with purified VLPs and the elicited immunity was compared to particles without Env (Gag_{p55}), to soluble monomeric Env_{gp120}, or to soluble trimerized Env_{gp140}. Only mice vaccinated with VLPs had robust anti-Env cellular immunity. In contrast, all mice had high titer anti-Env serum antibody (IgG). However, VLP-vaccinated mice had antisera that detected a broader number of linear Env peptides, had anti-Env mucosal IgA and IgG, as well as higher titers of serum neutralizing antibodies. VLPs elicited high titer antibodies that recognized linear regions in V4-C5 and the ectodomain of gp41, but did not

recognize V3. These lentiviral VLPs are effective mucosal immunogens that elicit broader immunity against Env determinants in both the systemic and mucosal immune compartments than soluble forms of Env.

3.3 INTRODUCTION

The ultimate goal of an AIDS vaccine is to elicit potent cellular and humoral immune responses that will result in enduring, broadly protective immunity (reviewed in [105] and [125]). While much effort has focused on elucidating the mechanisms and specificity of cellular immune responses, less is known about virus-specific antibody responses. It has been well established that cellular immune responses mediate viral control during the primary infection and maintain the viral set point in chronic infection [126]. However, administration of human monoclonal antibodies passively protects against virus exposure in the HIV-1/chimpanzee [127-128], SIV/monkey [129-130], and SHIV/monkey models [76, 131-134], demonstrating the ability of antibody alone to mediate protection against pathogenic virus infection. Thus, one goal of an AIDS vaccine should be to elicit robust and broadly reactive cellular and humoral immunity in both systemic and mucosal immune compartments, thereby maximizing the potential for protection from variant HIV-1 strains by different routes of exposure. A particle-based immunogen, such as a non-infectious virus-like particle (VLP), is a promising candidate for a safe and effective AIDS vaccine. VLPs are defined as self-assembling, non-replicating, non-pathogenic, and preferably genomeless particles that are similar in size and conformation to intact infectious virions. There are multiple combinations of viral proteins that may be used to

generate VLPs, however, they must contain Gag gene products in order to assemble and bud from cells. Env_{gp160} may also be processed and incorporated as trimeric spikes protruding from the surface of these particles.

The VLP used in this study is expressed from a virally regulated, multi-gene DNA plasmid. However, unlike many other VLPs currently being tested in AIDS vaccine research, our VLP-DNA plasmids can express multiple viral gene products from the same cell intracellularly and secrete these VLPs extracellularly from primate cells from a single DNA plasmid in vivo [135-136]. Other multi-gene-based VLP expression systems have been developed to express VLPs from DNA plasmids [137-140]. While these approaches successfully elicited cellular immunity against VLP antigens, these VLPs elicited limited binding antibody and little, if any, neutralizing antibodies, even after multiple vaccinations [137-138, 140-142]. In addition, low to undetectable cellular or humoral immune responses were observed at mucosal sites. The lack of a robust antibody responses may be due, in part, to the in vivo production of particles that were primarily retained intracellularly and therefore these viral antigens were processed for MHC class I presentation. Lastly, the lack of neutralizing antibodies in these studies may be a result of (1) the elicitation of low antibody titers or (2) the specific envelope chosen for incorporation into these particles. Over the past 20 years, multiple AIDS vaccine strategies have been developed, but each has failed to protect against disease (for reviews, see [105, 125, 143-145]). Early trials using monomeric HIV-1 envelopes, while eliciting high titer antibodies, failed to elicit antibodies capable of neutralizing HIV-1 in vitro. However, the role of Env appears critical for the induction of protective immunity in recent AIDS vaccines [146-147], which may point to other antibody and cellular immune mechanisms, such as antibody-dependent cytotoxicity (ADCC) [148-151], as important mediators of protection. Inclusion of Env in a multi-component AIDS

vaccine results in lower viral set points and higher CD4 counts following challenge compared to the same vaccines lacking Env [146-147]. Envelope on the native virion is predicted to form a trimer[152-154]. Thus, while neutralizing antibody responses appear to be an important component to elicit in an AIDS vaccine, they tend to recognize highly conformational epitopes on Env that may not be immunogenic in vivo. The inability of monomeric envelopes to elicit neutralizing antibodies is most likely due to differences in structure between monomeric forms of gp120 and oligomeric forms of envelope as they are expressed on the surface of a virus particle. Several vaccine strategies have incorporated an oligomeric/trimeric form of Env in order to elicit cross-reactive immunity that neutralizes viral infection [155-162]. The expression of trimeric Env on the surface of a particle does appear to elicit higher titers of neutralizing antibodies than soluble gp120 following intramuscular injection [163], even though much of the neutralizing antibodies were directed at membrane embedded cellular proteins. To overcome this problem, soluble trimers of Env have been developed. Soluble, stabilized Env trimers are hypothesized to mimic the trimeric structure of the envelope on the native virion and possibly induce conformationally dependent antibodies that recognize epitopes present only on native virion-associated envelopes. Several of these trimeric Env immunogens do elicit slightly higher titers of neutralizing antibodies than monomeric Env gp120 [157, 164-166]. Often, these oligomeric Env proteins are produced by eliminating the natural cleavage site recognized by cellular proteases [107, 162, 167-168]. The lack of elicited high titer, broadly reactive neutralizing antibodies by these immunogens is associated with the elicitation of primarily non-neutralizing antibodies [158, 169-170], because these uncleaved envelopes are in non-native forms or are processed through different cellular pathways than cleaved forms of Env [158-159, 171-172]. Therefore, in this study, mice were vaccinated with soluble envelopes (monomeric

Env gp120 or trimeric Env gp140) or trimeric envelopes that were membrane retained on an HIV-1 VLP to determine if the form(s) and presentation of envelope to the immune system influence the elicited immunity.

3.4 MATERIAL AND METHODS

3.4.1 DNA plasmids

The pTR600 vaccine plasmid [173] and the VLP expressing plasmid, pHIV-VLP, have been previously described [135]. Briefly, the pTR600 vector was constructed to contain the cytomegalovirus immediate-early promoter (CMV-IE) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription. The vector contains the Col E1 origin of replication for prokaryotic replication and the kanamycin resistance gene (kan^r) for selection in antibiotic media.

The pHIV-VLP plasmid [135] encodes for the following gene sequences: HIV-1_{BH10} gag-pol (pHIV_{BH10} nt 112–3626) (accession number M1564) and HIV-1_{ADA} vpu, env, rev, tat (nt 5101–8159). Briefly, the pHIV-VLP was constructed from two subclones encoding for the 5' end of the VLP gene insert (5'-ClaI and 3'-EcoRI) and a second subclone encoding for the 3' end of the VLP gene insert (5'-EcoRI and 3'-NheI). Oligonucleotides corresponding to the gag-pol sequences were used in a PCR reaction to amplify a fragment of DNA (5' PCR product) composed of the 5' untranslated leader sequence (105 nucleotides) and gag-pol sequences (ATG start codon of gag to the TAG stop codon at the 3' end of the reverse transcriptase sequence, pHIV_{BH10} nt 112–3626). Both of the 5' and 3' PCR products were cloned into pTR600 using

unique restriction enzymes sites (5' PCR product: ClaI and EcoRI and the 3' PCR product using EcoRI and NheI). The splice-acceptor sites were not mutated to allow for efficient mRNA processing of singly and multiply spliced messenger RNAs. The previously described safety mutations were introduced into the VLP vaccine insert by PCR-based mutagenesis (Stratagene, La Jolla, CA) to delete RT activity, RNase H activity, and packaging of RNA genome [135].

Plasmids expressing the HIV-1_{NL4-3} Gag gene products only, pGag_{p55}, were derived from codon-optimized sequences (phGag), as previously described [135, 174]. pGag_{p55} encodes for an immature, unprocessed HIV-1 Gag particle. The plasmids, pEnv_{gp120} and pEnv_{gp140} (FT), encode for soluble forms of monomeric Env_{gp120} or trimeric Env_{gp140} from the isolate ADA [156-157]. Each plasmid was amplified in *Escherichia coli* strain-DH5 alpha, purified using anion-exchange resin columns (Qiagen, Valencia, CA), and stored at -20 °C in dH₂O. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at a wavelength of 260 and 280 nm.

3.4.2 Transfections and expression analysis

The monkey fibroblast cell line COS (5×10⁵ cells/transfection) was transfected with 2 µg of DNA using 12% lipofectamine according to the manufacturer's guidelines (Life Technologies, Grand Island, NY). Supernatants (2 ml) were collected and stored at -20 °C. Cell lysates were collected in 500 µl of 1% Triton X-100 and stored at -20 °C. Quantitative antigen capture ELISAs were conducted according to the manufacturer's protocol (Perkin-Elmer Life Sciences, Boston, MA). For Western blot analysis, 3.3% of supernatant and 1.5% of the cell lysate were diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, CA), boiled for 5 min, and loaded onto a 10% polyacrylamide/SDS gel. The resolved proteins were transferred onto a nitrocellulose

membrane and incubated with a 1:10,000 dilution of polyclonal antisera using HIV-Ig to detect HIV-1 antigens diluted in PBS containing 0.05% Tween 20 and 5% nonfat dry milk. After extensive washing, bound human antibodies were detected using a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-human antiserum, followed by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

3.4.3 Purification of virus-like particles and soluble envelopes

Supernatants from COS cells, transiently transfected with plasmid expressing Gag or VLPs were pelleted via ultracentrifugation (100,000× g through 20% glycerol, weight per volume) for 2 h at 4 °C. The pellets were subsequently resuspended in PBS and overlaid onto 20–60% sucrose gradients (11 steps, 4% increments) and ultracentrifuged for 17 h at 100,000×g at 4 °C. Eleven fractions (20–60%, 1 ml, weight per volume) were collected top to bottom from the gradient, and the proteins were precipitated with equal volumes of 20% trichloroacetic acid (TCA) and subjected to SDS-PAGE and immunoblotting. The viral proteins were detected by primary antisera via Western blot. Determination of the amount of Gag and Env protein on the particle was determined by ELISA, as previously described [140]. Soluble monomeric Env_{gp120} or trimeric Env_{gp140} was purified from the supernatant of transiently transfected COS cells by a lectin column and resuspended in 0.9% saline for inoculation.

3.4.4 Immunization of mice

Female BALB/c mice (n=6, 5–7 weeks old) were immunized at weeks 0, 3, and 6 with purified VLPs (40 µg total protein) and co-inoculated with phosphorothioate CpG oligodeoxynucleotides

(CpG ODNs, 10 µg each) via the nares. Each CpG ODN (ODN-1: 5'-TCCATGACGTTCTGACGTT-3', ODN-2: 5'-TGACTGTGAACGTTTCGAGATGA-3') [175-179] was synthesized and purified by high-pressure liquid chromatography (Sigma-Genosys, The Woodlands, TX, USA). The CpG ODNs were resuspended in sterile dH₂O (2 µg/µl) and stored at -80 °C. Each purified VLP preparation was analyzed for purity by silver staining of an SDS-PAGE and ~80–90% of the protein in the purified preparation was Gag gene products and therefore, mice were administered ~0.5–1.0 µg of Env per VLP immunization. Mice were administered VLPs in sterile PBS plus CpG ODNs into the nares of each mouse (30 µl total volume). Supernatants from primate cells transiently transfected with the vector, pTR600, were ultracentrifuged through 20% glycerol. For intranasal inoculation, the pellet was resuspended in sterile PBS plus CpG ODNs and inoculated into the naïve mice (equivalent volume compared to mice given a VLP inoculum). Mice vaccinated with Env_{gp140} or Env_{gp120} were given 1 µg or 10 µg of protein with the same concentration of CpG ODNs in PBS. Proteins were purified from the supernatant of transiently transfected cells with plasmids expressing a 6×HIS-tagged envelopes [156-157]. Gag_{p24} was purified by the same ultracentrifugation procedure as VLPs. Mice were administered the same amount of Gag_{p24} plus CpG ODNs to match the Gag_{p24} in the VLP inoculations (40 µg). Mice were housed in compliance with USDA regulations and were monitored daily for weight loss, behavior, and adverse reaction. Mice were partially anesthetized with xylazine (20 mg/ml) and ketamine (100 mg/ml) administered subcutaneously in the abdomen prior to immunization.

3.4.5 Collection of samples

Blood samples were collected by retro-orbital plexus puncture on day 1 and weeks 2, 5, and 8 post-immunization on anesthetized mice. Sera samples were collected by centrifugation (5000 rpm, 10 min) and stored at -20°C . Mucosal washings were collected at week 9 post-immunization. Vaginal lavages were collected by repeated rinsing of the vagina (200 μl) with sterile PBS. The lungs were excised and cut into small pieces using a sterile scalpel in sterile PBS (200 μl). The lung tissue pieces were centrifuged (12,000 rpm, 5 min), and supernatants were collected. A section of the intestines (1.3 cm) was aseptically removed and placed in sterile PBS (200 μl). The collected fecal samples were disrupted using a sterile pipet tip, vortexed (15 s), centrifuged (12,000 rpm for 5 min), and the product supernatants were collected. The protein concentration of the fecal supernatants was adjusted to a final concentration of 1 mg/ml with sterile PBS. All samples were stored at -80°C .

3.4.6 ELISpot assays

Spleens were harvested from vaccinated mice at week 8, and splenocytes were isolated for ELISpot assays, as previously described [156-157]. Briefly, splenocytes were depleted of erythrocytes by treatment with ammonium chloride (0.1 M, pH 7.4). Following thorough washing with PBS, cells were resuspended in RPMI medium with 10% fetal bovine serum (cRPMI). Cell viability was determined by trypan blue exclusion staining. The number of anti-Gag, anti-Pol, or anti-Env_{gp160}-specific murine IFN- γ (mIFN- γ) secreting splenocytes was determined by enzyme-linked immunospot (ELISpot) assay (R&D Systems, Minneapolis, MN, USA). Briefly, pre-coated anti-mIFN- γ plates were incubated (25°C for 2 h) with cRPMI (200

μl) and then were incubated with splenocytes (1×10^6 /well) isolated from vaccinated mice. Splenocytes were stimulated (48 h) with peptides (AIDS Reagent and Reference Program) representing the consensus clade B Gag or Pol regions. For Env, four pools of peptides were used, (1) a pool of 10 peptides representing the C1 region of Env (amino acids 32–79), (2) a pool of 4 peptides representing the V3 region of Env (amino acids 294–314), (3) a pool of 99 peptides representing the gp120 region (amino acids 52–455) or (4) a pool of 99 peptides representing the gp41 region (amino acids 444–840) of the consensus Group M Env protein. The V3 regions peptides were specific to the sequence for the isolates, MN, IIB, the subtype B, and the consensus B sequences. The subtype B and consensus B match the V3 loop of ADA. Additional wells of splenocytes were stimulated with PMA (50 ng)/ionomycin (500 ng) or were mock stimulated. In addition, IL-2 was added to all wells (10 units/ml). Plates were washed with PBS–Tween (3×) and were incubated (25 °C for 2 h) with biotinylated anti-mIFN-γ and incubated (4 °C for 16 h). The plates were washed and incubated (25 °C for 2 h) with streptavidin conjugated to alkaline phosphatase. Following extensive washing, cytokine/antibody complexes were incubated (25 °C for 1 h) with stable BCIP/NBT chromogen. The plates were rinsed with dH₂O and air dried (25 °C for 2 h). Spots were counted by an ImmunoSpot ELISpot reader (Cellular Technology Ltd., Cleveland, OH, USA).

3.4.7 Antibody response to VLP immunizations

Serum and mucosal samples were individually collected and tested for antibody (IgG or IgA) responses to Env by ELISA. HIV-1_{ADA} Env_{gp120} was purified from the supernatants collected from 293T cells transiently transfected with a plasmid that expressed the HIV-1_{ADA} Env_{gp120} from codon optimized gene inserts. Each well of a 96-well plate was coated with 50 ng Env_{gp120}

per well (4 °C for 16 h). Plates were blocked (25 °C for 2 h) with PBS containing Tween 20 (0.05%) and nonfat dry milk (5%) and then incubated with serial dilutions of each sample (sera or mucosal washings) (25 °C for 2 h). Following thorough washing in PBS–Tween 20 (0.05%), samples were incubated (25 °C for 1 h) with goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) diluted 1:5000 in PBS–Tween 20 (0.05%) and nonfat dry milk (5%). The unbound antibody was removed, and the wells were washed. Samples were incubated with TMB substrate (1 h), and the colorimetric change was measured as the optical density (O.D., 405 nm) by a spectrophotometer (Dynex Technologies, Chantilly, VA, USA). The O.D. value of naïve sera and sera from mice vaccinated with virus-like particles composed of only Gag gene products (no Env) was subtracted from the samples using antisera from vaccinated mice. Results were recorded as the arithmetic mean \pm the standard deviation (SD). In order to measure the level of HIV-1 Env-specific IgG subtypes, biotinylated goat anti-mouse IgG1 or IgG2a (1:5000) antibodies were used to detect Env-antibody complexes.

3.4.8 Mapping of specific antibodies by poly-L-lysine peptide ELISA

Antibody reactivity to HIV peptides was determined using a poly-L-lysine (PLL) peptide ELISA, as previously described [180-182]. Briefly, the wells of Immulon 1B microtiter plates (Dynex Corporation, McLean, VA) were coated with 2 µg per well of PLL (Sigma, St. Louis, MO) diluted in 0.05 M sodium bicarbonate buffer (pH 9.6) and incubated for 1 h at 25 °C. Peptides were diluted to 50 µg/ml in 0.05 M sodium bicarbonate buffer (pH 9.6) were then fixed onto PLL-coated wells by incubating 50 µl per well overnight at 25 °C. After ~16 h, all wells were washed and blocked with 100 µl of glycine (1 M) followed by 0.5% nonfat dry milk/0.5% gelatin for 1 h each at room temperature. Serum was diluted 1:3000 in 5% nonfat dry milk and

incubated for 1h at room temperature. The wells were extensively washed and incubated with goat anti-mouse IgG-HRP diluted 1:5000 in 5% nonfat dry milk for 1 h at 25 °C. Specific reactivity was detected by the addition of 100 µl TMB substrate (Sigma, St. Louis, MO, USA). The reaction was stopped by the addition of 50 µl of sulfuric acid (1 N) and read as O.D. at 450 nm. Antibody reactivity was then reported as the O.D. at 450 nm at 1:3000 dilution of sera.

3.4.9 Pseudovirus preparation

HIV-1 viral cores were pseudotyped with envelopes representing primary R5 isolates (ADA, YU-2, CAAN5342.A2, PVO.4, and THRO4156.18) or R5X4 isolates (89.6). Briefly, 293T cells were transfected with 5 µg of Env encoding plasmid and 10 µg of an Env-deficient HIV-1 backbone vector (pSG3Δenv) using Lipofectamine 2000 (Invitrogen) [183]. Virus-containing supernatant was collected 48 h post-transfection, clarified of cellular debris by centrifugation (14,000 rpm for 10 min) and stored at -80 °C in 1 ml aliquots. TCID₅₀ was determined using 5-fold dilutions of virus. Eight hours prior to infection, TZM-bl cells were plated at a concentration of 1×10^4 cells per well. Cells were incubated with serial dilutions of virus for 48 h at 37 °C in 5% CO₂. Cell lysates were harvested and incubated (20 min) in lysis buffer (25 mM Tris phosphate, pH=7.8, 2 mM DTT, 2 mM 1-2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). Infectivity was determined by measuring the relative light units (RLU) using a Femtomaster FB12 Luminometer (Zylux, Maryville, TN). The TCID₅₀ was calculated from the RLU values.

3.4.10 Neutralization assay

Antisera from vaccinated mice were tested for the ability to neutralize virus infection in vitro using TZM-bl cells indicator cells [183-184]. These cells express human CD4 (hCD4), human CCR5 (hCCR5), human CXCR4 (hCXCR4), and a luciferase reporter driven by the HIV-1 LTR. TZM-bl cells were cultured in cDMEM with 10% fetal calf serum (10%) (Atlanta Biologicals, Atlanta, GA, USA). TZM-bl cells were plated at a concentration of 1×10^4 cells per well and incubated in DMEM with 10% FCS (8 h). Equal volumes of serial dilutions of each antiserum and pseudovirus were incubated for at least 1 h at 37 °C. Media was removed from the TZM-bl cells and replaced with the antiserum/pseudovirus mixture. The cells were then incubated for 48 h at 37 °C in 5% CO₂. Cell lysates were harvested and incubated in lysis buffer (20 min). Virus neutralization by mouse antiserum was determined by measuring the relative light units (RLU) using a Fentomaster FB12 Luminometer (Zylux, Maryville, TN). Neutralization by naïve sera and sera from mice vaccinated with virus-like particles composed of only Gag gene products (no Env) or MuLV Env was subtracted from the RLU from assays using antisera from vaccinated mice. All samples were tested in duplicate.

3.5 RESULTS

3.5.1 In vitro expression and particle purification of virus-like particles

An HIV-1 virus-like particle expressing plasmid was constructed from a proviral genome, as previously described [135]. The full-length HIV-1 VLP genome expressed Gag, protease (PR),

reverse transcriptase (RT), Vpu, Tat, Rev, and Env gene products to produce a PR-processed, but immature particle. A second VLP was expressed from a Gag only codon-optimized sequence that produced an unprocessed, immature particle. Plasmids expressing monomeric Env_{gp120} or trimeric Env_{gp140} envelopes have been previously described [157]. Expression of VLPs was determined in vitro following transient transfection of primate (COS) cells with each DNA plasmid. Cell culture supernatants and lysates containing Gag gene products were detected following SDS-PAGE and Western blot [135]. Particles were purified from the supernatants of transiently transfected cells by ultracentrifugation (20–60% sucrose gradient), and collected fractions were analyzed for particle composition and stability. VLPs banded in the same sucrose fractions (32–40% sucrose, 1.14–1.18 g/cm³), as previously described for wild-type virions (Fig. 6) [185-189].

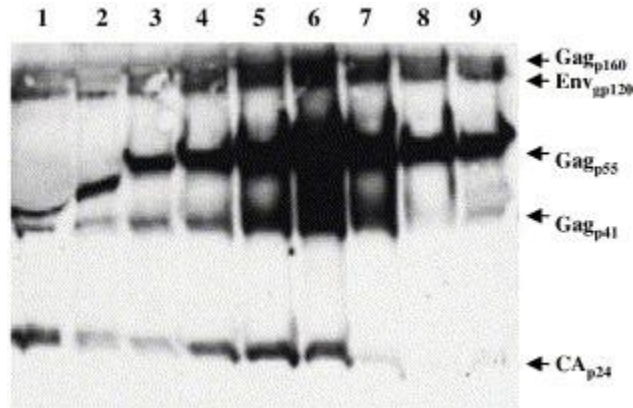


Figure 6 Virus-like particle production by sucrose density gradient ultracentrifugation.

Supernatants from COS cells transiently transfected with HIV VLP expressing DNA were concentrated through a 20% glycerol cushion and then subjected to 20–60% (w/v) sucrose density gradient-equilibrium gradient ultracentrifugation. Fractions were collected from top to bottom, precipitated with 10% TCA, and proteins were detected by Western blot analysis. HIV VLP. Percent of each sucrose fraction lane 1, 20%; lane 2, 24%; lane 3, 28%; lane 4, 32%; lane 5, 36%; lane 6, 40%; lane 7, 44%; lane 8, 48%; lane 9, 52%. All samples were electrophoresed through a 10% polyacrylamide/SDS gel and transferred to a PVDF membrane or silver stained. Proteins were immunoblotted with HIV-1 Ig (1:10,000) and mouse anti-human IgG (1:10,000) and visualized by enhanced chemiluminescence. Arrows indicate bands of HIV-1 antigens.

3.5.2 Cell-mediated immunity elicited by VLPs

Mice (BALB/c) were vaccinated intranasally with (1) purified HIV-1 VLPs, (2) Gag_{p55} only particles, (3) purified soluble trimerized Env_{gp140}, or (4) soluble monomeric Env_{gp120}, at day 1 and weeks 3 and 6. Collected splenocytes were stimulated in vitro with peptides specific for HIV-1 Gag, Pol or Env. Mice vaccinated with the HIV-1 VLPs had a robust cell-mediated immune response against Gag, Pol and Env. Four pools of Env peptides were used to detect splenocyte responses (mIFN- γ) specifically against the C1 region, the V3 region, a pool

representing the gp120 region of Group M consensus Env, or a pool representing the gp41 region of Group M consensus Env. Mice vaccinated with soluble Envs had a reduced cellular response, as measured by mIFN- γ production, compared to mice vaccinated with HIV VLPs (Fig. 7). Cellular responses against Gag elicited by HIV VLPs were statistically similar to the responses elicited by Gag only particles. Age-matched mice, as well as splenocytes from mice vaccinated with VLPs and stimulated with an irrelevant peptide or unstimulated, had few mINF- γ secreting splenocytes (0–7 spots) following in vitro re-stimulation (data not shown).

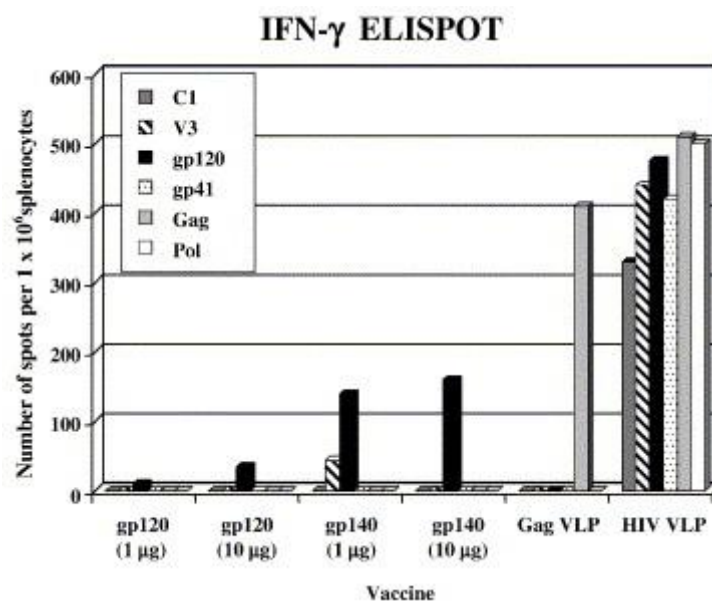


Figure 7 Elicitation of interferon- γ producing splenocytes.

ELISpots were performed on isolated splenocytes from vaccinated mice collected at week 8. Cells (1×10^6) were stimulated independently with peptides representing four different regions of Env: (a) the first constant region of gp120 (C1), (b) the V3 loop region of Env (V3), (c) the entire gp120 region (gp120), or (d) the entire gp41 region (gp41). Peptides in the gp120 and gp41 peptide pools were 15mers overlapping by 11 amino acids. The C1 and V3 pools are described in the Materials and methods. Splenocytes were also stimulated independently with pools of peptides representing Gag or Pol regions. Following stimulation, cells were assayed for mINF- γ . Nef peptides were used as a non-specific negative control. Splenocytes stimulated with PMA/ionomycin had colonies too numerous to count.

3.5.3 VLP-bound envelope efficiently elicits anti-Env antibody

All mice vaccinated with HIV VLPs or soluble envelopes had anti-Env antibodies against gp120 following the first inoculation, which increased after each subsequent vaccination. Mice vaccinated with soluble envelopes were administered a 10- to 100-fold higher dose of envelope than mice vaccinated with membrane-bound envelope on the surface of the VLPs (Fig. 8A). However, even though the amount of Env on the VLP was similar to the 1 µg dose of soluble envelope, the VLPs elicited a log higher titer of anti-Env antibodies (Fig. 8B). Mice vaccinated with VLPs had higher IgG2a titers compared to IgG1, indicating a T helper type 1 immune response. In contrast, mice vaccinated with soluble Env_{gp140} or Env_{gp120} elicited a predominantly IgG1 antibodies, indicating a dominant T-helper 2 response.

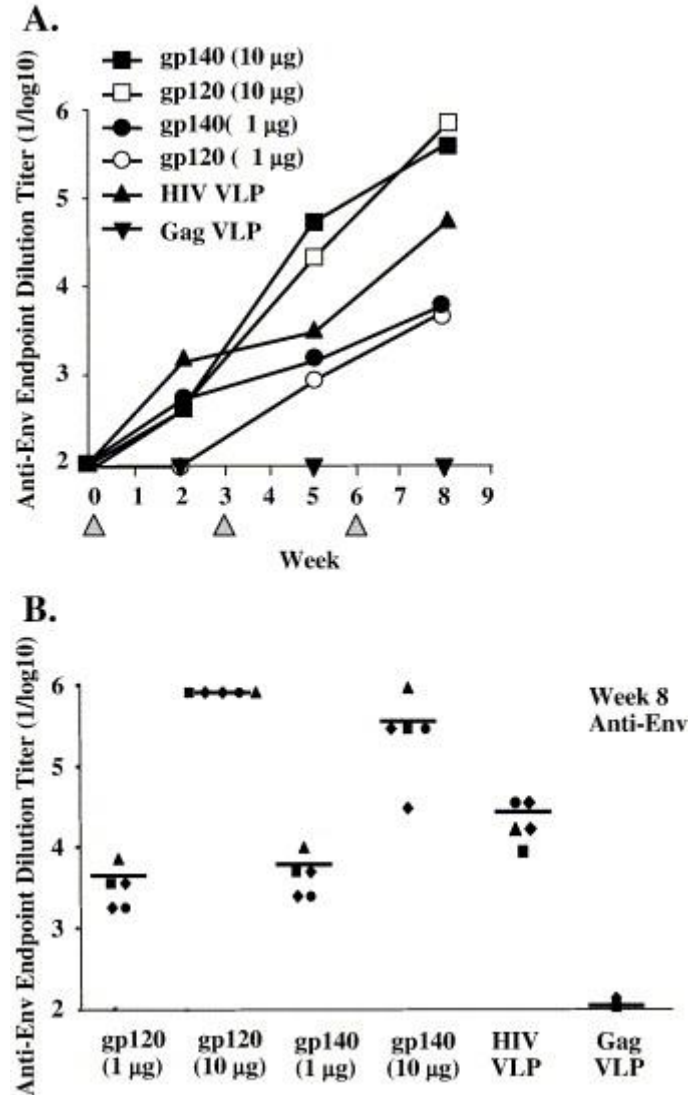


Figure 8 Anti-Env serum antibodies.

(A) Total serum anti-gp120 Env IgG. Serum samples were collected prior to immunization (week 0) and 2 weeks after each vaccination (weeks 2, 5, and 8). Each symbol represents the average endpoint anti-Env serum antibody titer ($n = 6$). (B) Symbols represent each individual mouse anti-Env serum IgG endpoint titer. The line represents the average titers (week 8).

Both anti-Env IgG and IgA were detected in mice vaccinated with VLPs (Fig. 9). The highest titers were detected in the lungs, but anti-Env antibodies were also detected in the secretions of the vagina and intestine, albeit at lower titers (Fig. 9). Mice vaccinated with soluble

envelopes (10 μ g) generally had statistically lower titers than VLP-vaccinated mice and mice vaccinated with a 1 μ g dose of soluble Envs had undetectable mucosal anti-Env antibodies.

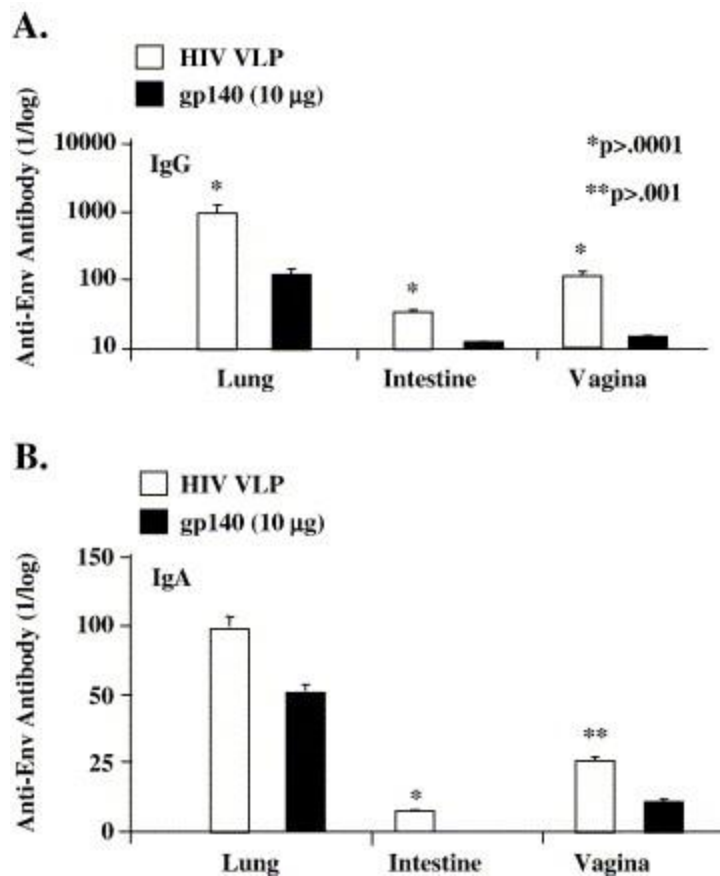


Figure 9 Total anti-Env IgG and IgA in the mucosa.

Anti-gp120 Env antibody titers were determined in three mucosal tissues (lungs, intestines, and vagina) at week 8 post-vaccination from HIV VLP- or gp140 (10 μ g)-vaccinated mice. (A) IgG. (B) IgA. Each bar represents the average anti-Env antibody titer ($n = 6$). The statistical significance of the difference between groups was calculated by the Student's t test.

3.5.4 Virus-like particles broaden the anti-Env antibody responses

Serum samples were assayed for the ability to recognize individual, overlapping peptides representing the entire Env_{gp160}. Sera collected from all vaccinated mice recognized greater than

50% of all the peptides at low levels (<0.2 O.D, less than 3-fold over background). However, mice vaccinated with VLPs recognized a larger number of Env-specific peptides at higher titers compared to mice vaccinated with soluble envelopes (Fig. 10). Sera from mice vaccinated with soluble envelopes bound fewer than 9 peptides with a titer that was greater than 3-fold over background (non-specific peptides, as well as sera from naïve mice) (Figs. 10 A and B). In contrast, collected sera from HIV-1 VLP-vaccinated mice recognized 40 peptides with a titer that was 3-fold over background (Fig. 10 C). Sera from VLP-vaccinated mice recognized peptides in both gp120 and gp41 and sera from mice vaccinated with gp140, which contained the ecto-domain of gp41, did not recognize any of the peptides representing gp41.

Even though peptides from regions throughout Env were recognized by sera from VLP-vaccinated mice at high titers, there did appear to be hotspots of recognition. Peptides overlapping the cleavage site, representing the V4-C5 regions in gp120 and the amino terminal region of gp41, were specifically recognized by this serum (Fig. 10). Interestingly, sera from VLP-vaccinated mice also recognized peptides in gp41 that overlap the highly cross-reactive Kennedy epitope. A few specific peptides were recognized by sera from VLP-vaccinated mice to regions in C2. Lower titer antibodies (2- to 3-fold over background; <0.2 O.D.) recognized several peptides in V1, V2, and C2, but few high titer antibodies (greater than 3-fold over background; >0.2 O.D.) recognized peptides in V1 or V2 and no peptides were detected to V3. Sera from mice vaccinated with Env_{gp120} did detect three V3-specific peptides at high titers, but Env_{gp140}-vaccinated mice did not. In contrast, numerous peptides in the V1, V2, C2, and V3 regions were recognized by antisera at lower titers (less than 3-fold) from mice vaccinated with soluble envelopes (data not shown).

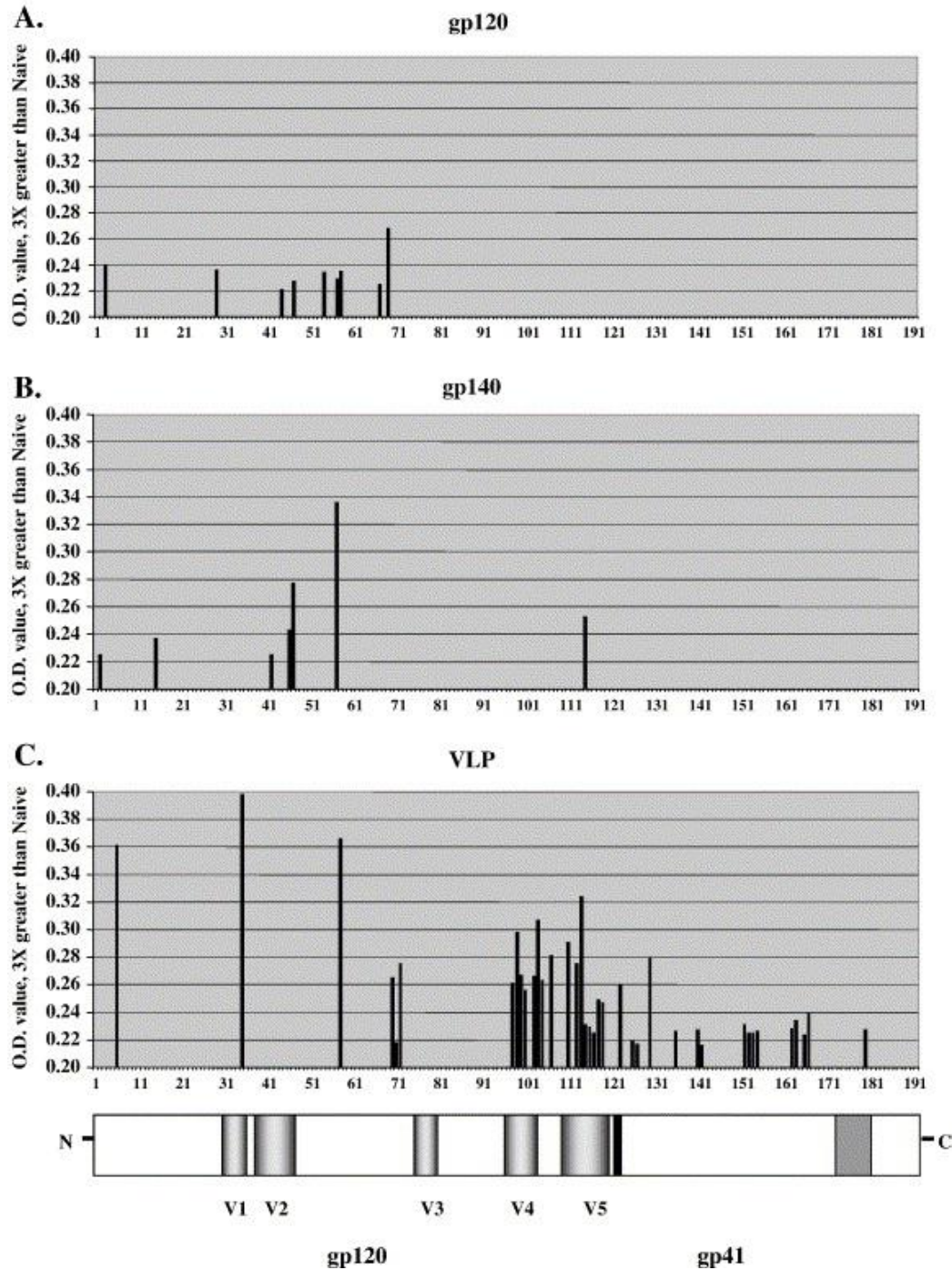


Figure 10 Location in Env corresponding to high titer induced antibodies recognizing linear epitopes.

Relative peptide location in the Env amino acid sequence detected by antiserum from each vaccinated mouse group that had an O.D. value greater than 3-fold over background. Background was measured as the O.D. value against irrelevant peptide (a Nef peptide) or the

reactivity of sera from naïve, age-matched mice. Vaccine: (A) Env_{gp120} (1 µg), (B) Env_{gp140} (1 µg), (C) HIV VLP. Bottom schematic displays regions of Env, the variable regions (gray), and the transmembrane domain (black).

3.5.5 Neutralization activity

Sera were examined for the ability to neutralize virus infection in vitro [184] against both neutralization-sensitive and neutralization-resistant viral isolates (Table 1). Sera from mice vaccinated intranasally with HIV-1 VLPs had antibodies that neutralized both homologous and heterologous virus isolates (50% inhibition, no IC₉₀ values detected). The R5 isolates representing early transmission (CAAN5342.A2, PVO.4, and THRO4156.18) were more difficult to neutralize than YU-2 or ADA. The dual-tropic isolate, 89.6, was consistently the more easily neutralized isolate and the only isolate neutralized with sera from mice vaccinated with soluble envelopes (Table 1). Sera from mice vaccinated with Gag_{p24} only did not neutralize any of the viral isolates (data not shown). In addition, the antibodies in the sera from VLP-vaccinated mice were specific for Env, since the sera from these mice neutralized viruses with HIV-1 Env and did not neutralize viruses pseudotyped the MuLV Env (Table 1).

Table 1 Neutralization titers

Vaccine	Virus ^a						
	89.6	ADA	YU-2	CAAN ^b	PVO.4	THRO ^c	MuLV
HIV VLP	80 ^d	160	80	40	40	40	10
gp140	80	10	10	10	10	10	10
gp120	80	10	10	10	10	10	10

^a Antisera from vaccinated mice were incubated with each virus pseudotyped with the indicated viral envelopes.

^b CAAN=CAAN5342.A2.

^c THRO=THRO4156.18.

^d IC₅₀ titers from sera collected at week 10. Inhibition of viral infection of TZM-bl cells was assessed by the additional reduction in infectivity beyond the background of sera from age-matched naïve mice.

3.6 DISCUSSION

In this study, HIV-1 virus-like particles were expressed from a single gene insert lacking the sequences for LTRs, IN, Vpr, Vif, and Nef that was engineered with safety mutations in CA_{p24} and RT to prevent viral RNA packaging and reverse transcriptase activity [135]. The VLPs were produced from a virally regulated modified proviral genome that resulted in a self-assembling, non-replicating, nonpathogenic, genomeless particle that was similar in size and conformation to intact virions. These VLP immunogens can be administered as purified particles or expressed in vivo from DNA plasmids/viral vectors (for reviews, see references [190-191]). Only a few systems have been developed that utilize the viral regulatory mechanisms of a lentiviral genome to express particles in primate cells [135, 192].

Mice inoculated intranasally with these HIV-1 VLPs elicited robust cellular responses to Gag, Pol, and Env gene products (Fig. 7). Soluble forms of Env were administered at doses 10- to 100-fold higher than the Env on the VLP, yet elicited few cellular responses. Both VLPs and soluble envelopes did elicit robust anti-Env serum antibody (Fig. 8), however, mice vaccinated with VLPs elicited antibodies that recognized a broader number of Env-specific peptides at higher titers than sera from mice vaccinated with soluble envelopes (Fig. 10). In addition, only mice vaccinated with VLPs had high mucosal anti-Env antibodies (Fig 9). Despite similar anti-Env IgG serum titers (Fig. 8), only mice vaccinated with VLPs had antibodies that blocked viral infection of neutralization-resistant viruses in an in vitro neutralization assay (Table 1). Therefore, the presentation of native forms of envelope in the context of a viral membrane to the immune system elicited significantly broader immunity than soluble forms of envelope following mucosal vaccination.

The goal of this study was to determine if envelope, presented to the immune system on the surface of a viral particle, is a more effective immunogen than soluble forms of Env. The role of Env appears critical for the induction of protective efficacy by recent AIDS vaccines [146-147]. Non-human primates immunized with Gag only vaccines do not control viral infection as well as the same vaccine that included Env [146]. However, the specific immune mechanism(s) associated with Env in those vaccines has not been determined, but most likely is associated with anti-Env cellular and antibody responses.

Envelope on the native virion is predicted to form a trimer [152, 193-195]. Therefore, our laboratory and others have used soluble, oligomeric/trimeric forms of Env to mimic the proposed native trimeric structure of Env on the viral membrane in order to elicit cross-reactive immunity and induce conformationally dependent antibodies that recognize epitopes present only

on the native virion-associated envelopes [155, 157-162, 196-198]. These uncleaved oligomeric Env proteins do elicit marginally higher neutralizing antibody titers than monomers of Env_{gp120} when administered parenterally [157, 164-166]. The lack of high titer, broadly reactive neutralizing antibodies elicited by these immunogens is associated with the elicitation of primarily non-neutralizing antibodies [159, 169-170].

Even though all the vaccines in this study elicited serum anti-Env antibodies, the envelope-bound VLPs elicited higher titers of both mucosal antibodies (Fig. 9) and serum antibodies that recognized a broader range of linear Env epitopes following intranasal administration (Fig. 10). Similar titers were elicited using a monomeric Env_{gp120} or trimeric Env_{gp140} expressed in a DNA prime/protein boost by intramuscular injection [157, 199]. These results are supported by a recent study from Grundner et al. [197] that demonstrated Env_{gp140} cleavage-defective trimers (similar to the Env_{gp140} trimers used in this study) are more effective at generating neutralizing antibodies when presented on a solid phase proteoliposomes, indicating that the Env_{gp140} trimer presented on the surface of a lipid bilayer is a more effective immunogen than nonmembrane-bound Env trimers. However, there are most likely considerable differences between cleaved Env_{gp160} trimers on our VLPs and uncleaved Env_{gp140} trimers, even when presented on a lipid membrane.

Linear antibodies elicited in VLP-vaccinated mice were directed to regions in gp120 outside V3, similar to sera from mice vaccinated with Env_{gp140} proteoliposomes [197]. The role of the V3 region in eliciting neutralizing antibodies has been controversial. Even though V3-specific antibodies can neutralize T-cell-adapted (TCLA) HIV-1 isolates by recognizing a linear site (GPGR/Q), they do tend to be strain-specific [200-201]. V3-specific antibodies are often elicited using Env_{gp120} monomers due to sequence accessibility on gp120, but this region is less

accessible in oligomeric forms of Env [200, 202-203] and may be less accessible in primary HIV-1 isolates [154, 200, 204]. Even though few V3-specific linear epitopes were recognized at high titers by sera from mice vaccinated with VLPs, we cannot rule out that the antibodies recognized conformational epitopes in V3. Sera from both soluble Env- and VLP-vaccinated mice were tested in a concanavalin A (Con A) capture ELISA. All serum samples retained high endpoint dilution titers to gp120 (>1:10 000), indicating that the sera contained antibodies that recognized both conformational epitopes on Env, as well as linear epitopes. The elicited anti-Env antibodies recognized a cluster of peptides just before (V4-C5 region in gp120) and just after (ectodomain of gp41) the cleavage site.

At lower titers, sera from both soluble Env- or VLP-vaccinated mice recognized peptides throughout gp120, including the V1 and V2 regions, however, few of the V1 and V2 peptides were detected at high titers. Three immunodominant peptides in V2 were detected in all VLP- and soluble Env-vaccinated mice (peptide 47, representing NDNTSSYRLISCNTS and peptides 57–58, representing ILKCNDKKFNGTGPCTNVS). Even though antibodies directed to V1, V2, and/or V3 have been shown to be potent in neutralizing virus in *in vitro* infection assays [205-207], the role of these antibodies in protection is unclear and controversial [205-206, 208-212].

An interesting set of peptides detected by sera from VLP-vaccinated mice was the highly cross-reactive Kennedy epitope (residues 731–752, SRFPDRPEGTEEEGGERDRDRS) located in a region classified as the intra-cytoplasmic domain (ICD) of gp41 [213]. It has been widely assumed that the ICD is located completely within the inner leaflet of the viral or cellular lipid bilayer. However, neutralizing antibodies are directed to the Kennedy epitope [213-217], and at least part of the Kennedy epitope, in the context of intact virions, is reactive with specific monoclonal antibodies and susceptible to protease digestion, consistent with ICD virion or

infected cell exposure. Based on antigenic properties and a theoretical structural analyses of Env_{gp41} ICD sequences from various HIV-1 clades (357 sequences), Hollier and Dimmock recently proposed that ICD sequences potentially form a tail loop structure supported by three β -sheet membrane spanning domains [218]. Therefore, antibodies directed at this region in Env_{gp41} would only be elicited by Env_{gp160} molecules found in particle-based vaccines or from Env_{gp160} expressed in vivo from DNA plasmids or viral vectors.

We chose to use a mouse model for these studies primarily because this small animal model allows for detailed analysis of both cellular and humoral immunity induced by these VLP vaccines. However, one limitation of sera collected from mice is the intrinsic high titers of non-specific background observed in *in vitro* neutralization assays. Despite these limitations, sera collected from VLP-vaccinated mice were able to neutralize both neutralization-sensitive and neutralization-resistant viral isolates (Table 1). Mice vaccinated intranasally with either soluble monomeric Env_{gp120} or trimeric Env_{gp140} had neutralizing antibodies to only 89.6. Even though our laboratory and others have demonstrated that trimeric Env_{gp140} elicits modest levels of neutralizing antibodies, the lack of neutralizing antibodies from soluble Env-vaccinated mice may be a result of the route of inoculation [mucosal (intranasal) vs. parenteral (intramuscular/intradermal)]. The neutralizing capacity of sera collected from VLP-vaccinated mice was specific for the HIV-1 envelope, since this serum did not neutralize virus pseudotyped with MuLV Env (Table 1). These HIV-1 Env-specific neutralizing antibody titers were above the background neutralizing antibodies directed against cellular monkey proteins embedded in the VLP membrane, since sera from mice vaccinated with Gag only particles (no envelope) elicited few antibodies, which were subtracted from the final analysis. Recently, Hammonds et al. recorded high levels of neutralizing antibodies to cellular embedded proteins in VLP-vaccinated

guinea pigs following intramuscular injection [163], but significant neutralizing titers were still present following depletion of the non-specific neutralizing antibodies. The reasons for the differences in neutralizing capacity in the sera from these two studies are unclear, but may be due to the route of inoculation (intranasal vs. intramuscular), the amount of Env on each of the particles, the animal species used (mice vs. guinea pigs), or the phenotype of the envelope isolates used [183]. These differences are unlikely a result of the neutralization assay used, since both studies used the same TZM-bl assay [183]. No neutralizing antibodies were detected in the lung washes from our vaccinated mice, which may be directly due to the mouse model used or the low titers of mucosal antibodies detected (Fig. 9). Future studies in larger rodents (rabbits or guinea pigs) or non-human primates will be necessary to determine if VLPs elicit neutralizing antibodies in mucosal tissues.

Why might the VLPs described in this study be such effective mucosal immunogens? Compared to particulate antigens, intranasal vaccination of soluble proteins, in the absence of an adjuvant, induces low or undetectable immune responses in rodents and primates [219]. Intranasal immunization of VLPs induces both systemic and mucosal immunity. Soluble antigens can penetrate the nasal epithelium and directly interact with dendritic cells, macrophages and lymphocytes and then these antigens are transferred to posterior lymph nodes [220]. In contrast, VLPs are most likely phagocytosed by microfold epithelial cells (M cells) in the nasal lumen and then directly deposited to the NALT (nasal associated lymphoid tissue) via M cell transcytosis [81], which preferentially drains into lymph nodes. This process induces strong local (NALT) and distant immune responses in both peripheral and mucosal immune compartments [221]. Soluble antigens bypass the NALT and are directly fed into superficial lymph nodes by antigen presenting cells in the nasal lumen resulting in a lower local immune response [221]. Therefore,

VLP immunogens directly interact with the mucosal immune system to elicit high titer immunity. Overall, the expression of Env in any HIV/AIDS vaccine may elicit a variety of potentially effective protective immunity depending on the structure of the protein presented to the immune system. Following mucosal administration, the membrane-bound Env on the surface of a particle appears more effective at eliciting immunity that specifically recognizes Env on the native particle compared to the immunity elicited by soluble monomers or trimers of Env.

3.7 ACKNOWLEDGMENTS

This research was supported by National Institute of Health Grant AI51213 to T.M.R. The authors thank Franklin Toapanta and Heather Grieser for their technical assistance, and Kelly Stefano Cole and Ronald C. Montelaro for their helpful discussions. The following peptides and the following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl cells (also called JC57BL-13) (#8129), HIV-1ADA (#416), HIV-Ig (#3957, from NABI and NHLBI).

4.0 SPECIFIC AIM 2

Comparison of the breadth of cellular immunity generated by Consensus, Polyvalent and Monovalent HIV-1 virus-like particle vaccines

This chapter was modified with permission from:

Sean P. McBurney and Ted M. Ross

Comparison of the breadth of cellular immunity generated by Consensus, Polyvalent and Monovalent HIV-1 virus-like particle vaccines

Vaccine. 2009 Jul 9;27(32):4337-49

4.1 ABSTRACT

Envelope (Env) sequences from human immunodeficiency virus (HIV) strains can vary by 15-20% within a single clade and as much as 35% between clades. Previous AIDS vaccines based upon a single isolate often could not elicit protect immune responses against heterologous viral challenges. In order to address the vast sequence diversity in Env sequences, consensus sequences were constructed for clade B and clade C envelopes and delivered to the mouse lung mucosa on the surface of virus-like particles (VLP). Consensus sequences decrease the genetic difference between the vaccine strain and any given viral isolate. The elicited immune responses were compared to a mixture of VLPs with Envs from primary viral isolates. This polyvalent vaccine approach contains multiple, diverse Envs to increase the breadth of epitopes recognized by the immune response and thereby increase the potential number of primary isolates recognized. Both consensus and polyvalent clade B Env VLP vaccines elicited cell-mediated immune responses that recognized a broader number of clade B Env peptides than a control monovalent Env VLP vaccine in both the systemic and mucosal immune compartments. All three clade C Env vaccine strategies elicited similar responses to clade C peptides. However, both the consensus B and C Env VLP vaccines were more effective at eliciting cross-reactive cellular immune responses to epitopes in other clades. This is the first study to directly compare the breadth of cell-mediated immune responses elicited by consensus and polyvalent Env vaccines.

4.2 INTRODUCTION

The development of an effective AIDS vaccine has been hampered by the intrinsic diversity among circulating populations of HIV-1 in various geographical locations. There is a need to develop vaccines that can elicit enduring protective immunity to variant HIV-1 strains. Based on viral genetic distances and positions in phylogenetic trees, HIV-1 is divided into three separate groups: M, O, and N [10]. Group O infections are limited to central Africa and Group N infections are found in a small number of patients in Cameroon [222]. The vast majority of viral isolates are designated as Group M and they are responsible for the continuing worldwide AIDS pandemic. Due to the diversity in amino acid sequences between isolates within Group M, this group is further subdivided into nine subtypes or clades (A-D, F-H, J and K) [10]. The diversity between isolates in different clades can vary by as much as 35%.

The ultimate goal of an AIDS vaccine is to elicit potent cellular and humoral immune responses that will result in enduring, broadly protective immunity. Over the past 20 years, a number of potential AIDS vaccines have elicited immune responses that decreased viral set-points and maintained CD4⁺ T cells in vaccinated animals, but sterilizing immunity has not been achieved [223-226]. Often, this limited protection occurs when the challenge virus is homologous to the viral proteins of the vaccine [223-226]. However, these artificial challenge models are not reflective of human infections and therefore the diversity of HIV-1 isolates requires vaccine designs that elicit broadly reactive immunity.

The greatest diversity is localized to the viral envelope glycoproteins which may reflect its primary role in eliciting host immune recognition and responses that result in progressive evolution of the envelope proteins during persistent infection. Interestingly, while Env variation is widely assumed to be a major obstacle to AIDS vaccine development, there are very few

experimental data in animal or human lentivirus systems addressing this critical issue. One method previously used to address Env variation in an AIDS vaccine is a polyvalent vaccine strategy [106-110, 224]. These vaccines consist of a mixture of divergent isolates of the same antigen administered simultaneously. Polyvalent vaccines function by presenting a wide range of epitopes that cover a majority of individual strains. Vaccines for pneumococcus, poliovirus, and influenza virus have used this strategy [105]. Polyvalent vaccines increase the strength and breadth of humoral immune responses compared to monovalent vaccines [107-109, 114-116, 118, 227-228]. The increase in the number of immunogens, below a certain threshold, does not result in immune interference [229].

More recently, centralized sequences have been developed as an alternative vaccine strategy to address viral sequence diversity. These artificial sequences are designed using computational methods to minimize the distance between a vaccine strain and primary wild-type isolate. The three main strategies for developing centralized vaccines are center of the tree, ancestral, and consensus (for review see [230-231]). Each of these designs has advantages for vaccine development. Consensus sequences minimize the degree of sequence dissimilarity between vaccine immunogens and circulating virus strains by creating artificial sequences based upon the most common amino acid in each position in an alignment [63, 232-236]. Recently, vaccine strategies utilizing consensus or ancestral HIV-1 Gag and Env sequences have proven potent inducers of CTL activity [86-89, 233]. These consensus Envs form native structures and facilitate infection via the CCR5 co-receptor [85, 90].

In this study, monovalent, polyvalent and consensus Env vaccines were compared for the ability to elicit broadly reactive cellular immune responses following vaccination in mice. Each set of Env vaccines was generated from clade B or clade C envelope sequences. HIV-1 virus-

like particles (VLP) were used to deliver these Env sequences to the immune system in their native trimeric structure on the surface of a viral particle and the elicited immune responses were compared for the breadth of Env reactivity.

4.3 MATERIALS AND METHODS

4.3.1 Envelope gene sequences

The wild-type HIV-1 subtype B and C and consensus full-length *env* gene sequences were derived from the most common amino acids found at each location within the Env gene from over 200 isolates for each clade (Los Alamos National Laboratory; www.lanl.gov). Consensus genes were generated from the derived consensus sequences by GeneArt (Burlingame, CA) in Contemporary subtype B and C *env* genes were cloned by polymerase chain reaction (PCR) amplification from Env_{gp160} plasmids obtained from the AIDS Reagent and Reference Program (National Institutes of Health). Primers were designed to insert a 5' *XhoI* site and a 3' *NheI* site to facilitate cloning into the VLP vector. The nucleotide sequences of the *env* genes are available under accession numbers PVO.4 (AY835446), AC10.0.29 (AY835446), RHPA4259.7 (AY835447), SC422661.8 (AY835441), Du151.2 (AY835441), CHN19 (AF268277), and ZM214m.PL15 (DQ388516).

4.3.2 DNA Plasmids

The pTR600 vaccine expression plasmid [237] and the HIV-1 VLP expressing plasmid, have been previously described [238]. Briefly, the pHIV-wtVLP_{ADA} plasmid encodes for the following gene sequences: HIV-1_{BH10} *gag-pol* (pHIV_{BH10} nt 112–3626) (accession number M1564) and HIV-1_{ADA} *vpu, env, rev, tat* (nt 5101–8159). Safety mutations were engineered into Gag to prevent viral RNA packaging [239-240] and RT to prevent reverse transcriptase and RNase H activity (pHIV-VLP_{ADA}) [23, 241-242]. Each VLP was expressed from a cytomegalovirus immediate-early promoter (CMV-IE) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription. Consensus Env VLPs were constructed by substituting ADA *env* with the consensus *env* sequences from the consensus clade B and C envelopes (LANL database). The primary isolate VLPs were constructed by substituting ADA *env* with the primary *env* sequences. Plasmids expressing the HIV-1_{NL4-3} Gag gene products only, pGag_{p55}, were derived from codon-optimized sequences (phGag), as previously described [238, 243]. pGag_{p55} encodes for an immature, unprocessed HIV-1 Gag particle. Each plasmid was amplified in *Escherichia coli* strain-DH5 alpha, purified using anion-exchange resin columns (Qiagen, Valencia, CA), and stored at -20°C in dH₂O. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at a wavelength of 260 and 280 nm.

4.3.3 Purification of virus-like particles.

Supernatants from COS cells, transiently transfected with plasmid expressing Gag or VLPs were purified via ultracentrifugation (100,000 X g through 20% glycerol, weight per volume) for 4 h at 4°C. The pellets were subsequently resuspended in PBS and stored at -20°C until use. Protein concentration was determined by Micro BCA™ Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA).

4.3.4 Expression analysis

For Western blot analysis, 1 µg of purified VLP was diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, CA), boiled for 5 min, and loaded onto a 10% poly-acrylamide/SDS gel. The resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad) and incubated with a 1:500 dilution of HIV-1_{IIIB} rabbit anti-gp120 polyclonal sera (Advanced Biotechnologies Inc, Columbia, MD) in PBS containing 0.05% Tween 20 and 5% nonfat dry milk. After extensive washing, bound rabbit antibodies were detected using a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit anti-serum and enhanced chemi-luminescence (Amersham, Buckinghamshire, UK). The Western blots were scanned and densitometry was determined using ImageJ software (<http://rsbweb.nih.gov/ij/>). Expression levels were normalized to ConB VLP as it had the highest expressions levels.

4.3.5 Immunization of mice.

Female BALB/c mice (5–7 weeks old) were immunized at weeks 0, 4, and 8 with purified VLPs (20 µg total protein) and co-inoculated with phosphorothioate CpG oligodeoxynucleotides (CpG ODNs, 5 µg each, total of 10 µg) via the nares in a total volume of 30 µL. Each CpG ODN (ODN-1: 5'-TCCATGACGTTCTGACGTT-3', ODN-2: 5'-TGACTGTGAACGTTTCGAGATGA-3') [244-248] was synthesized and purified by high-pressure liquid chromatography (Sigma-Genosys, The Woodlands, TX, USA). The CpG ODNs were resuspended in sterile dH₂O (2 µg/µl) and stored at -20°C. The composition of each purified VLP preparation is approximately 80-90% Gag gene products and therefore, mice were administered ~0.5-1.0 µg of Env per VLP immunization. Mice were housed in compliance with U.S.D.A. regulations and were monitored daily for weight loss, behavior, and adverse reaction. Mice were partially anesthetized with xylazine (20 mg/ml) and ketamine (100 mg/ml) administered subcutaneously in the abdomen prior to immunization.

4.3.6 Collection of samples.

Blood samples were collected by retro-orbital plexus puncture on weeks 0, 2, 6, and 10 post-immunization on anesthetized mice. Sera samples were collected by centrifugation (5,000 rpm, 10 min) and stored at -20°C. Spleens were harvested from vaccinated mice at week 10, and splenocytes were isolated for ELISpot assays, as previously described [249-250]. Briefly, splenocytes were depleted of erythrocytes by treatment with ammonium chloride (0.1 M, pH

7.4). Following thorough washing with PBS, cells were resuspended in RPMI medium. Cell viability was determined by trypan blue exclusion staining.

4.3.7 Antibody response to VLP immunizations.

Serum samples and lung washes were individually collected and tested for antibody (IgG) responses to homologous VLP by ELISA. Each well of a 96-well plate was coated with 50 ng per well of the respective VLP (4°C for 16 hr). Plates were blocked (25°C for 2 hr) with PBS containing Tween 20 (0.05%) and nonfat dry milk (5%) and then incubated with serial dilutions of each sample (25°C for 2 hr). Following thorough washing in PBS-Tween 20 (0.05%), samples were incubated (25°C for 1 hr) with biotinylated goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) diluted 1:5000 in PBS-Tween 20 (0.05%) and nonfat dry milk (5%). The unbound antibody was removed, and the wells were washed. Samples were incubated with TMB substrate (1 hr), and the colorimetric change was measured as the optical density (O.D., 405nm) by a spectrophotometer (BioTek Instruments, Winooski, VT, USA). The O.D. value of naïve sera was subtracted from the samples using antisera from vaccinated mice. Results were recorded as the arithmetic mean \pm the standard deviation.

4.3.8 Neutralization assay.

Antisera from vaccinated mice were tested for the ability to neutralize virus infection *in vitro* using TZM-B1 cells indicator cells [251-252]. These cells express human CD4 (hCD4), human CCR5 (hCCR5), human CXCR4 (hCXCR4), and a luciferase reporter driven by the HIV-1 LTR.

TZM-B1 cells were cultured in cDMEM with 10% fetal calf serum (10%) (Atlanta Biologicals, Atlanta, GA, USA). Infectivity was determined using serial dilutions of antisera with cells in complete, non-selective media in the presence of DEAE dextran (20 µg/ml) (25°C for 1hr). Cell lysates were harvested in lysis buffer (25mM Tris phosphate, pH=7.8, 2mM DTT, 2mM 1-2-diaminocyclohexane-N, N, N', N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) (48 h) and then clarified by centrifugation. Virus neutralization by mouse antisera was determined by measuring the relative light units (RLU) using a Femtomaster FB12 Luminometer (Zylux, Maryville, TN). Neutralization by naïve sera and sera from mice vaccinated with virus-like particles composed of only Gag gene products (no Env) was subtracted from the RLU from assays using antisera from vaccinated mice.

4.3.9 ELISpot assays.

The number of anti-Gag and anti-Env specific murine IFN- γ (mIFN- γ) secreting splenocytes and lung cells was determined by enzyme-linked immunospot (ELISpot) assay (R & D Systems, Minneapolis, MN, USA). Briefly, pre-coated anti-mIFN- γ plates were incubated (25°C for 1 h) with cRPMI (200 µl) and then were incubated with splenocytes (5×10^5 /well) or lung cells (1×10^6 /well) isolated from vaccinated mice. Cells were stimulated (48 h) with peptides (15mers overlapping by 11 amino acids) representing the consensus clade B HIV-1 Gag, consensus clade B, or consensus clade C Env proteins (NIH ARRRP). IL-2 was added to all wells (10 units/ml). Control wells were stimulated with PMA(+) (50 ng)/ionomycin (500 ng) or were mock stimulated(-). Plates were washed with PBS-Tween (3X) and were incubated (37°C for 48 h; 5% CO₂) with biotinylated anti-mIFN- γ and incubated (4°C for 16 h). The plates were washed and

incubated (25°C for 2 h) with streptavidin conjugated to alkaline phosphatase. Following extensive washing, cytokine/antibody complexes were incubated (25°C for 1 h) with stable BCIP/NBT chromogen. The plates were rinsed with dH₂O and air dried (25°C for 2 h). Spots were counted by an ImmunoSpot ELISpot reader (Cellular Technology Ltd., Cleveland, OH, USA).

4.3.10 CD4 cell depletion

CD4 cells were depleted from single cell suspensions of splenocytes from individual mice. Depletions were completed using the CD4 (L3T4) microbeads and an autoMACS Separator per manufacturer's instructions (Miltenyi Biotec Inc., Auburn, CA).

4.4 RESULTS

4.4.1 Selection of wild-type and design of consensus B or C *env* gene sequences.

The aim of this study was to compare the breadth of immune responses elicited by wild-type (individually or in a mixture) or consensus envelope sequences. The consensus (Con) B or C Env sequences were generated by selecting the most common amino acid at each position of a full-length Env alignment derived from 200 subtype B or C viruses deposited in the 2005 HIV Sequence Database. Each set of subtype sequences was also supplemented with *env* sequences used in a standardized neutralizing panel [183, 253].

The wild-type sequences used to design monovalent or polyvalent vaccines were selected based upon 4 criteria: route of transmission, weeks after transmission, place of transmission, and neutralization phenotypes. All Envs were chosen from isolates from patients that received the virus via sexual transmission. Five of the seven isolates were transmitted by heterosexual transmission (M-to-F or F-to-M) and two isolates by M-to-M transmission (Table 2). Two of the clade B viruses were isolated from patients living in United States, one in the Caribbean, and one in Europe, whereas the three clade C isolates were collected from patients living in Africa and Asia. All of the isolates used in the polyvalent vaccines were collected in the acute phase of infection and were neutralization resistant [183, 253]. Fig. 11 A and B depicts an alignment of Con B and Con C Env_{gp160} sequences and the deduced protein sequences of the contemporary wild-type *env* sequences. One of the strains from each polyvalent vaccine was used as a monovalent vaccine control. Primary and consensus clade B and C Envs efficiently bound to human CD4, were CCR5-tropic and facilitated infection with similar efficiency (data not shown).

Env sequences were cloned into the HIV-1 VLP expression plasmid that contained the HIV-1_{BH10} *gag-pol* and HIV-1_{ADA} *vpu, env, rev, tat* [238]. Following purification, each particle contained similar amounts of envelope (Fig. 12). The expression of Env_{gp160} from each VLP vaccine was determined to ensure that similar doses of Env were on the particle surface and delivered with each VLP.

Table 2 Characteristics of envelopes used in the virus-like particle vaccines

Vaccine	Isolate name	Geographic location of patient	Mode of transmission ^a	Length of infection ^b
Gagp24	–	–	–	–
Consensus B (Con B)	–	–	–	–
Polyvalent B (Poly B)	PVO.4	Italy	M–M	4 weeks
	RHPA4259.7	USA	M–F	<8 weeks
	SC422661.8	Trinidad	F–M	4 weeks
	AC10.0.29	USA	M–M	4 weeks
Monovalent B	PVO.4	Italy	M–M	4 weeks
Consensus C (Con C)	–	–	–	–
Polyvalent C (Poly C)	DU151	South Africa	M–F	6 weeks
	ZM214M	Zambia	F–M	<13 weeks
	CHN19	China	–	–
Monovalent C	DU151	South Africa	M–F	6 weeks

^a Direction of sexual transmission: M–M male to male, M–F male to female, F–M female to male.

^b Length of infection is defined as time between last negative HIV-1 test and first positive HIV-1 test.

ConB	MLERVKGIRKNYQH-LWRWGTMLLGMLMICSAAEKLWVTVYYYGVPVWKEATTTLFCASDA
AC10	.--..RET.....LW.K..M.....V.QT..... <u>N</u>
PVO.4	.--..T.....S...M.....E.....
RHPA	.--..M.....K.....WL.....DQ..... <u>N</u>
SC42	.--..RET.R.W...W.K.....T.....

ConB KAYDTEVHNWATHACVPTDPNPQEVVLENVTFENFNMWKNMVEQMHEDIISLWDQSLKP
 AC10 . . . N E D
 PVO.4 . . . N G
 RHPA A H
 SC42 . . . E . . K D G

VI

ConB	CVKLTPLCVTLNCTDLMNATNTTN--SSSGEKME-KGEIKNCSFNITTSIRDKVQKEYA
AC10S...NVGN ¹ NTS... <u>N</u> .RWD..... <u> </u>NM...M..Q..
PVO.4 <u> </u> .S..R.. <u> </u>PTV..RVI.K.MM..V.. <u> </u>V..D...RM..V..
RHPA <u> </u>V.S.. <u>I</u> .R.....VDNTT.EK.MK.. <u> </u>V.SG.....
SC42 <u> </u>ELRNGTYAN.....VTVT.....A.....T..

V2

ConB	LFYKLDVVPI-D---ND <u>NT</u> ---SSYRLISC <u>NT</u> SVITQACPKVSFEPIPIHYCAPAGFA
AC10 E.EGK. <u>N</u> .S..SFTD.....T.....
PVO.4	...RP....Q.HTIE. <u>N</u> .IEN <u>NTT</u>I.....T.....
RHPA	.L...I.Q.....ND <u>NT</u> SHRD <u>NT</u>I.....
SC42	..R.....NNHG.SSS <u>NY</u> .N...N.....

ConB ILKCNDDKKFNGTGPTNVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVIRSENFTNNAKTI
 AC10 L...K..._...K..._...K..._...S...R...
 PVO.4 ..._...S..._..._...R...I..._...
 RHPA ..._..._..._..._..._...V.N
 SC42 ...N..._...K..._..._...D...

V3

ConB	IVQL <u>N</u> ESVEIN <u>C</u> TRPN <u>N</u> TRKSIHIGPGRAFYT <u>T</u> GEIIGDIRQAHC <u>N</u> ISRAKW <u>M</u> NTLKQI
AC10	... <u>T</u> ... <u>K</u> .I... <u>G</u> ... <u>D</u> ... <u>Q</u> N.. <u>N</u> ...
PVO.4	... <u>K</u> T... <u>S</u> ... <u>A</u> . <u>D</u> ... <u>L</u> .. <u>E</u> . <u>K</u> .. <u>Y</u> .
RHPA	... <u>Q</u> .. <u>H</u> .. <u>N</u> ... <u>A</u> . <u>K</u> ... <u>E</u> . <u>Q</u> ...
SC42	... <u>D</u> . <u>I</u> ... <u>G</u> . <u>T</u> ... <u>V</u> .. <u>V</u> ... <u>V</u> .. <u>L</u> . <u>S</u> ... <u>S</u> .. <u>V</u> .

	V4
ConB	VKKLREQFGNKTI ^{FN} QSSGGDPEIVMHSFNCGGEFFYC ^{NT} TQLF ^{NT} STWN--VNGTWN ^{NN}
AC10	AE..... <u>R</u> <u>N</u>T..A.....AE.....Y..A...IS ^I -
PVO.4	ST..... <u>I</u> .. <u>G</u>T..... <u>K</u>DANG ^C .GCDE
RHPA <u>K</u> .. <u>A</u> .AP.....N..... <u>K</u> .. <u>T</u> .. <u>STW</u> ^S ...T
SC42	.T.....F..... <u>I</u> ...HGT

	V5
ConB	TEGNDT-ITLPCR ^Q IKQ ^I INMWQEVGKAMYAPP ^I RQ ^I RCSS ^N ITG ^L LL ^L TRDGG--NMNT ^N
AC10	GG. ^Q K.N.I.....F.....S.....RG. ^Q TD.
PVO.4	SD. ^Q N.....V.....K.L.K.T.....A.....
RHPA	EGS. ^S .V.....R.....Q...K.....VDT.K
SC42	.VS. ^Q K...I.....-...NS
	gp120 gp41
ConB	ETE ^I IRPGGGDMRD ^N WRSELYKYKV ^V KIEPLGVAPT ^K AKRRV ^V QREKRAVG-IGAMFLGF
AC10	Q...F..V....KN.....R.....I..R.....L....
PVO.4	..--F.....Q.....I..R.R.....TL.....
RHPA	..--F.....N.K.....R.....
SC42	T...F.....I.....R.....T.....
ConB	LGAAGSTMGAASMTLTVQARQLLSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARVLA
AC10L.....
PVO.4V.....K.....M.....
RHPAI.....L.....S.....
SC42V.....L.....
ConB	VERYLKDQQLLGIWGCSGKLICTTTVPWN ^A SW ^S N ^K SLDEIWD ^M TWMEWEREID ^N YTS ^L I
AC10R.....A.....V..N..R.V.D.E.....Q.D...S.....
PVO.4	I.....A.....T.....FNK.....G..
RHPAA.....Q.T.G.....Q.....D..
SC42R.....R.....N...T...ND...D..N...
ConB	YTLIEESQNQQEKNEQELLELDKWASLWNWFDITNWLWYIKIFIMIVGGLIGLRIVFAVL
AC10A.....N.....N..E.....V.....
PVO.4	.N.L.....D..A...E.....S..K.....
RHPA	.N.L.....A.....S..S..H.....M.....V.....
SC42	.N.L.DA.....H.....I.....
ConB	SIVNRVRQGYSP ^L SFQ ^T RLPAPRGPD ^R PEGIEEEGG ^E RD ^R DRSGRLVDGFLALIWD ^D DL ^R S
AC10H...Q.....G.....S.....N...I..I....
PVO.4H..TS.....GG.....P.....I..V....
RHPAF.....SA...V.V.V...N
SC42H...R.E.....T.G...K...V...H.....
ConB	LCLFSYHRLRDL ^L LIVTRIVELLGRRGWEVLKYWNLLQYWSQELKNSAVSLLNATAIAV
AC10H.....I.....I.....I.....I.....
PVO.4	.F.....T....L.....A.....R.....D.....
RHPAT.....A.....K.....D.I.....
SC42T.....G.....A.....DTI....
ConB	AEGTDRVIEVVQ ^R ACRAILHIPRRIRQGLERALL
AC10	G..K..I..AFRSIF.....T.....S..
PVO.4I.....TF...I.....L..
RHPAI..LL..IF..F...T.....Q
SC42A.R.IFIGV.....F.....

B.

```

ConC      MLERVGILRNCQQWWIWGILGFWMLMICNVMGNLWVTVYYGVPVWKEAKTTLCASDAK
CHN19     .--..T..RK.Y.HL.R..TMLLGM....SAAE.....
DU151     .--..E....Y.....T.....V.....
ZM214     .--....M.....GV.....R.....

```

```

ConC      AYETEVHNVWATHACVPTDPNPQEMVLENVTFNFNMWKNDMVDQMHEDIISLWDQSLKPC
CHN19     ...K...........N....V.....
DU151     ..DK......I...........
ZM214     ...K......L...........N.....

```

V1

```

ConC      VKLTPLCVTLNCRNNVTNNAT---NNNNN-TMKEEIKNCSFNITTELDRDKKQKVYALFY
CHN19     .....E.-.SSG....Y.ETY......A..V..R..T.....
DU151     .....T-----PA..Y.---S.HG.M......T..I..R..A....
ZM214     .....S-.NI.E.SIDF.VTS.IS...M......KVNS....NRREH...

```

V2

```

ConC      RLDIVPLNEKMNSNYSSD-YRLINCNTSAITQACPKVSFDPIPIHYCAPAGYAILKCNNK
CHN19     .....K..SEN..EY......T......T......D.
DU151     KP.V...RREEN.GTGE..I......S.T......T......
ZM214     K....Q..DEG.DS..--......T.K.....E......E

```

```

ConC      TFNGTGPCNNVSTVQCTHGIKPVVSTQLLLNGSLAEEEEIIIRSENLTNNAKTIIVHLNES
CHN19     ........H.......R......V......Q.
DU151     ..............N......I......K.
ZM214     ...S............K..M......Q.T.A

```

V3

```

ConC      VEIVCTRPNNNTRKSIRIGPGQTFYATGDIIGDIRQAHCNISEEKWNKTLQRVSEKLKEH
CHN19     ............KD...E....GK..A..
DU151     ......R.....E..N..E...KSS.TS..EQ.KK....
ZM214     .N.T.M..G...R.V.....E......KD...QI..N.RA..G..

```

V4

```

ConC      FPNKTIKFAPSSGGDLEITTHSFNCRGEFFYCNTSKLFNSTYMPNST-NN---TNTTITL
CHN19     ......E..S......I...G.....G.EG...SSSI..I
DU151     Y-....E.N.P.....V......T...S-----.NSDS....-.E....
ZM214     .HD....E.....G......TN..SR..-T.GSNS.VNI.SA....

```

ConC PCRIKQIINMWQEVGRAMYAPPIEGNITCKSNITGLLLTRDGGK-NDTNDTTETFRPGGGD V5
 CHN19 V.....-E........
 DU151 ..K.....K.................T...-I.....N
 ZM214 A.....I.....G.....A.....

	gp120	gp41
ConC	MRDNWRSELYKYKVVEIKPLGVAPTKAKRRVVEREKRAVGIGAVFLGFLGAAGSTMGAAS	
CHN19I...A.....	
DU151	.K.....E.....SK.....L...L.....	
ZM214I.....G.....	

ConC ITLTVQARQLLSGIVQQQSNLLRAIEAQQHMLQLTVWGIKQLQTRVLAIERYLKDQQLG
CHN19S.....L.....
DU151
ZM214N.....L.....A.....

ConC IWGCSGKLICTTAVPWNSSWSNKSQEDIWDNMTWMQWDREISNYTDTIYRLLEDSQNQQE
CHN19H.....R...E..N.....N.....
DU151 L.....I.....G.....
ZM214 L.....T.S.....VD...Q.....N..EI...V.....

ConC KNEKDLLALDSWKNLWNWFDITNWLWYIKIFIMIVGGGLIGLRIFAVLSIVNRVRQGYSP
CHN19 R.....N.....S.....R.....
DU151N.....G..A..K.....
ZM214 ..E.....K.D.....SK.....

ConC LSFQTLTPNPRGPDRLGRIEEEGGEQDRDRSIRLVNGFLALAWDDLRLSLCLFSYHRLRDF
 CHN19G.....KN.....N..R...L...L
 DU151S.....KN.....S.....L...L
 ZM214EL.....S.....H...L

ConC ILIAARAVELLGRSSLRGLQRGWEALKYLGSLVQYWGLELKKSAISLLDTIAIAVAEGTD
 CHN19 L..V..I.....R.WWN.LK..VQ...N..V...NAT.....
 DU151 ..VVT.....N...GG...R...K.F.....
 ZM214 ..T.S.....G...R.....V..T.....

ConC RIIELIQRICRAIRNIPRRIRQGFEAALQ
CHN19 .V..VV.GAY...LH.....
DU151 ..L.V.....H..I.....L
ZM214 .V..IA..FG.G.C.....

Figure 11 Generation of consensus (Con B and Con C) envelopes.

Alignment of the deduced protein sequence of full-length (unmodified) Con B Env with those of recently transmitted, contemporary subtypes B and C Env isolates. Sequences are compared to consensus gp160, with dots indicating sequence identity, and dashes indicating gaps introduced for optimal alignment. Potential N-linked glycosylation sites are in *italics*, **bolded** and underlined. The locations of the gp120/gp41 cleavage sites are indicated, as are the positions of the variable loops (V1–V5) and the transmembrane (TM) domain. (A) Con B. (B) Con C.

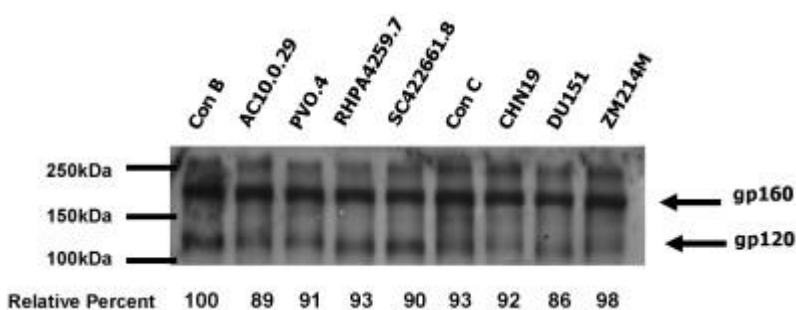


Figure 12 Envelope expression on virus-like particles.

(A) Purified virus-like particles were loaded with 1 μ g of total protein in each well in a SDS-polyacrylamide gel under reducing conditions and probed with rabbit anti-HIV_{IIIB} gp120 polyclonal sera. The level of envelope expressed in the VLP was determined by comparison of the density of the bands observed in the Western blot. Levels are shown as percent of envelope as compared to Con B.

4.4.2 Antibodies elicited by VLP vaccines.

Groups of mice (BALB/c) were vaccinated with either 1) the consensus Env VLPs (Con B or Con C) individually, 2) wild-type Env VLPs individually (monovalent: PVO.4 or DU151), or 3) a mixture of wild-type Env VLPs (Poly B or Poly C). As a control, viral particles composed of Gag_{p24} only (no Env) were administered. All vaccines elicited high-titer serum antibodies that recognized the VLP (1:50,000-1:100,000) at week 10 post-vaccination (Fig. 13A). In addition,

anti-VLP IgG antibodies were detected in the lung wash from all mice (Fig. 13B). Neutralizing antibodies were detected in the serum from all vaccinated groups (1:40-1:80). However, these titers were the same as those detected in mice vaccinated with Gag_{p24} particles.

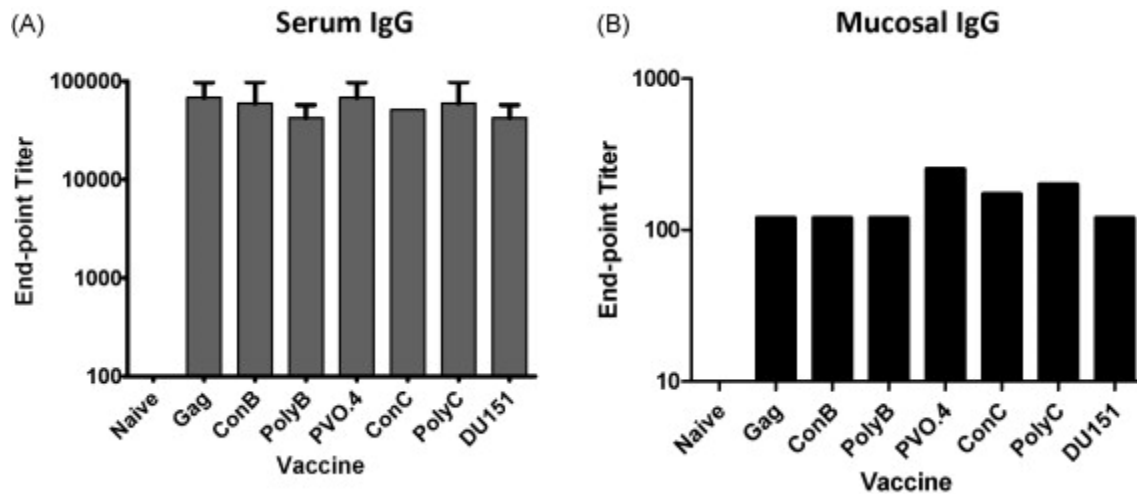


Figure 13 Antibody responses.

(A) Anti-VLP titers were determined by ELISA with 96-well plates coated with matched VLPs at 50 ng per well. Week 10 sera were tested starting at a dilution of 1:200. Endpoint was assigned as the dilution at which background was reached. (B) Week 10 lung washes were used to determine anti-VLP titers in the mucosa. Washes were tested starting at a dilution of 1:10. All responses are shown as the average titer for each vaccine group plus the standard deviation.

4.4.3 Elicitation of Cellular Immune Responses.

Splenocytes from mice vaccinated with either consensus Env VLPs and wild-type Env VLPs (monovalent or polyvalent) secreted IFN- γ following stimulation with Env specific peptides (Fig. 14A and B). No Env-specific immune responses were elicited in mice vaccinated with the Gag_{p24} particles. Pools of peptides representing each region of Env_{gp160} were matched to the subtype (*i.e.* subtype B peptides were used to stimulate cells from subtype B vaccinees). Anti-Env splenocyte responses were detected throughout all regions of Env in both clade B (300-800

spots per 10^6 splenocytes) and clade C (100-400 spots per 10^6 splenocytes). There was no statistical difference in the number of spots elicited between consensus Env VLPs and polyvalent or monovalent Env VLP vaccines in either subtype. Interestingly, there were differences in the cellular responses elicited in the lung mucosa (site of vaccination) compared to the responses detected in the spleen. All three vaccine strategies (consensus, polyvalent, and monovalent) elicited a high number of responses to the V3 region for both subtypes (Fig. 14C and D). However, mice vaccinated with Con B VLPs had additional responses to C1, C3, V4-C5, ectodomain of gp41 (gp41_{ecto}), the transmembrane (TM) and amino terminal region of the intracytoplasmic domain (gp41_{TM/ICD N'}). In contrast, mice vaccinated with Poly B VLPs had significant responses to only gp41_{TM/ICD N'} and the carboxyl region of the intracytoplasmic domain (gp41_{ICD C'}). The monovalent PVO.4 VLP elicited mucosal responses against V4-C5, gp41_{TM/ICD N'}, and gp41_{ICD C'} regions. Similar responses were observed using the subtype C VLP vaccines (Fig. 14D).

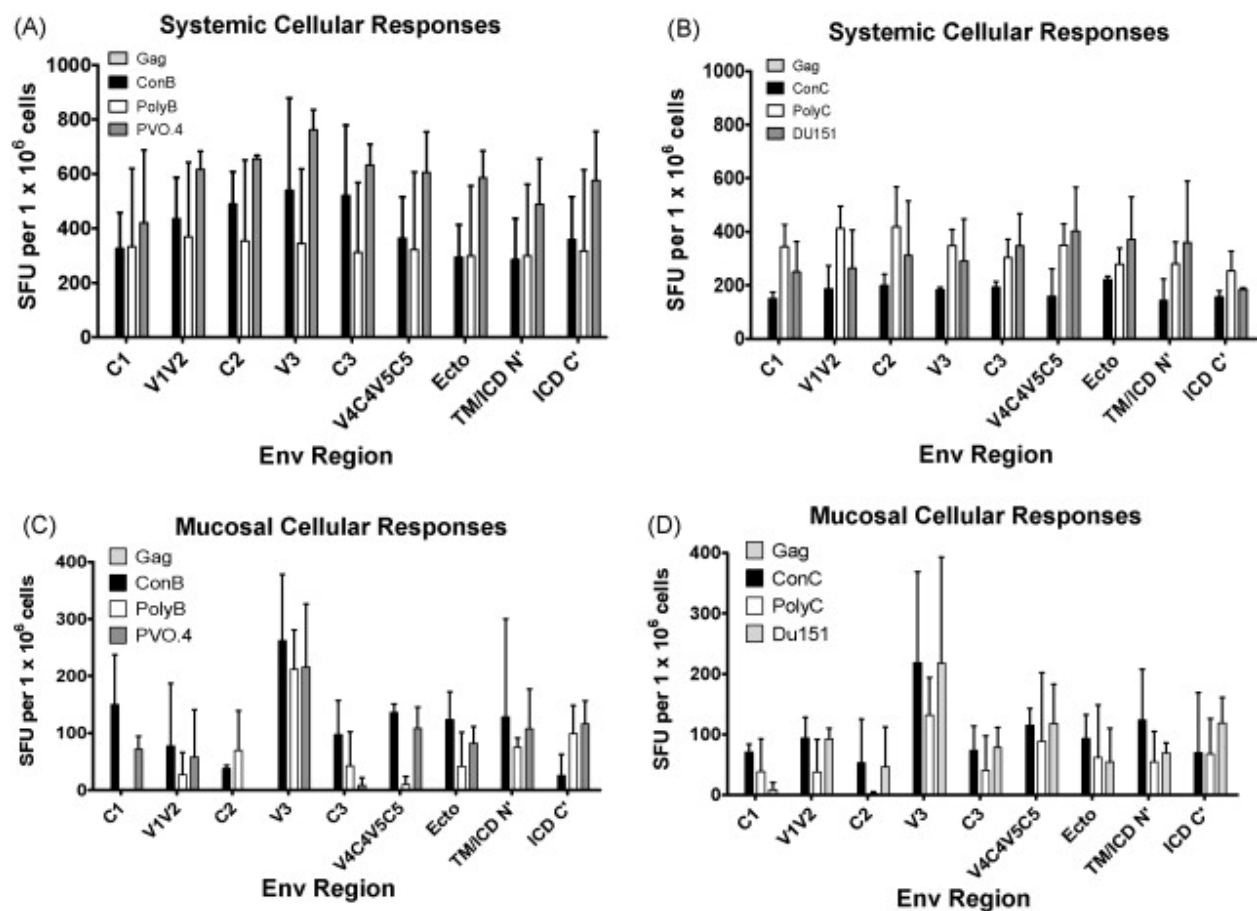


Figure 14 Anti-envelope cell-mediated immune responses.

T cell immune responses induced by consensus, polyvalent and monovalent clades B and C vaccines. Splenocytes (A and B) and lung cells (C and D) were isolated and stimulated with overlapping clade-matched envelope peptides. Peptides were separated into approximately equal sized pools of peptides representing the different regions of envelope. Responses are represented as SFU per million cells. The values of each column are the mean \pm standard deviation from individual animals (n=10).

4.4.4 Envelope Cellular Epitopes

Individual peptides responsible for eliciting cellular immune responses in each peptide set were identified using matrix format peptide pools and then confirmed by testing the peptides

individually [254]. Thirty-five individual peptides were identified in mice vaccinated with the Con B VLP against the subtype B peptide set and 35 cross-reactive peptides were identified against the subtype C peptide set (Fig. 15 and Table 2). Similar number of peptides was detected in mice vaccinated with the Poly B VLPs, however, almost no peptides were identified using the monovalent PVO.4 VLP. Interestingly, at least 15 individual peptides were identified by each subtype C VLP vaccinees against both subtype B and subtype C peptide sets. Mice vaccinated with the Poly C had similar number of identified peptides (15 against subtype B peptides and 17 against subtype C peptides). The monovalent DU151 VLP vaccinated mice responded to a comparable number of peptides as the Poly C against subtype B peptides (20), but to an increased number of identified subtype C peptides (32). Interestingly, mice vaccinated with the Con C VLP responded to twice as many individual peptides identified (50) from the subtype B peptide set as the subtype C peptide set (23).

The identified epitopes were mapped to each region of Env_{gp160} (Fig. 16). Since both subtype B and subtype C peptide contain overlapping peptides, it was important to determine the percent of the Env covered by these responses and not just the number of peptides identified. The Con B and Poly B VLP vaccines clearly demonstrated increased coverage of both subtype B and C peptide sets compared to the monovalent PVO.4 VLP (Fig. 16 and Table 3). Mice vaccinated with the Con B or Poly B VLP vaccines elicited cellular responses that covered 40-50% of the envelope with equal distribution throughout the protein. Mice vaccinated with Con C VLPs elicited similar coverage as the Con B VLP (~61%) as detected by the subtype B peptide set (Fig. 16), however, only half as much of Env was covered when the subtype C peptide set was used for analysis, with almost no recognition of the peptides in the Env_{gp41}. Both the Poly C and monovalent DU151 VLP vaccines elicited responses that recognized less of the envelope

than the Con C vaccine when tested using the subtype B peptide set. However the monovalent DU151 VLP had more coverage of the Env than Con C or Poly C VLPs when splenocytes were stimulated using the subtype C peptide set.

To determine the subpopulation of T cells elicited by these vaccines, splenocytes were depleted of CD4⁺ T lymphocytes. Less than 10% of the remaining splenocytes were CD4 positive. The most reactive common epitopes from both peptide sets were tested against the CD4⁺ depleted splenocytes. This subset of peptides elicited similar numbers of IFN- γ producing spots to each of the epitopes tested (Fig. 17). Upon the depletion of CD4⁺ T lymphocytes, all immune responses were lost indicating that all epitopes were CD4 restricted.

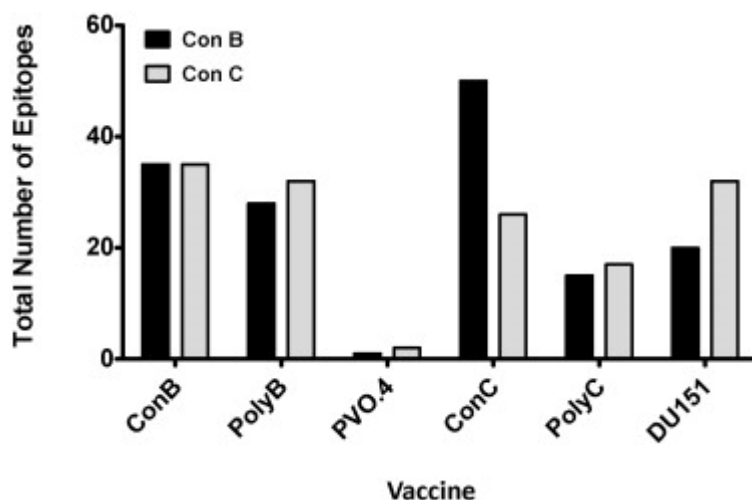


Figure 15 Identification of envelope-specific peptides.

Individual envelope peptides were identified by ELISpot using peptide pools in a matrix format. Epitopes were considered positive if they were at least two-fold higher than responses observed with Gag only vaccinations. Total number of epitopes recognized by each vaccine in both Con B and Con C envelope peptide sets.

Table 3 Env peptides individually identified by VLP vaccines.

Con B ^a																			Total
Con B ^b	8766	8774	9779	8787	8791	9779	8806	8807	8814	8818	8826	8834	8839	8847	8851	8859	8866	8867	35
	8874	8878	8886	8894	8897	8905	8909	8917	8925	8928	8936	8940	8948	8956	8957	8965	8969		
Con C ^b	9186	9187	9189	9190	9191	9193	9199	9201	9202	9203	9205	9215	9217	9219	9221	9231	9233	9249	35
	9253	9261	9265	9277	9278	9281	9282	9290	9293	9294	9306	9310	9322	9369	9373	9381	9385		
Poly B ^a																			
Con B ^b	8764	8769	8780	8782	8784	8793	8813	8823	8831	8855	8869	8875	8883	8891	8912	8915	8920	8929	28
	8931	8935	8941	8943	8944	8949	8951	8964	8972	8973									
Con C ^b	9185	9193	9197	9200	9205	9219	9223	9233	9245	9253	9257	9265	9274	9282	9286	9293	9294	9308	32
	9312	9332	9334	9341	9342	9345	9346	9354	9368	9370	9372	9374	9382	9395					
PVO.4 ^a																			
Con B ^b	8971																		1
Con C ^b	9364	9368																	2
Con C ^a																			
Con B ^b	8763	8766	8768	8777	9779	8782	8784	8789	8791	8797	8799	8813	8815	8821	8823	8837	8839	8849	50
	8850	8851	8852	8857	8859	8866	8868	8873	8875	8878	8880	8883	8886	8888	8899	8902	8904	8911	
	8912	8919	8928	8935	8940	8941	8943	8948	8957	8959	8964	8969	8971	8972					
Con C ^b	9188	9190	9202	9226	9250	9255	9257	9262	9275	9277	9279	9281	9284	9286	9291	9293	9304	9308	23
	9310	9320	9322	9377	9395														
Poly C ^a																			
Con B ^b	8777	8789	8791	8817	8837	8839	8849	8877	8879	8911	8926	8928	8966	8968	8969				15
Con C ^b	9199	9208	9210	9239	9245	9261	9274	9290	9301	9305	9330	9332	9363	9365	9379	9381	9394		17
DU151 ^a																			
Con B ^b	8763	8791	8807	8819	8821	8833	8839	8849	8853	8855	8862	8865	8877	8882	8883	8896	8911	8913	20
	8927	8939																	
Con C ^b	9191	9207	9214	9222	9231	9238	9239	9243	9245	9251	9253	9262	9267	9269	9270	9291	9293	9299	32
	9301	9303	9311	9327	9332	9334	9340	9351	9356	9359	9380	9388	9392	9394					

^a Vaccine group.

^b Env peptide set, numbers represent the NIH AIDS Research and Reference Reagent Program catalog number for each identified peptide.

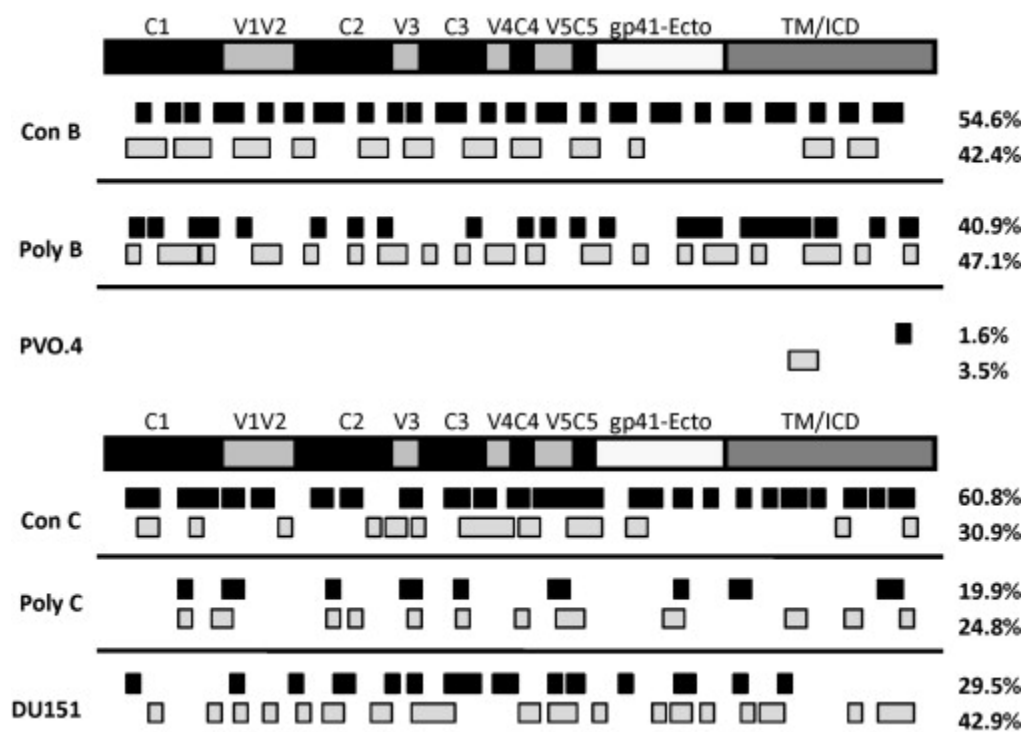


Figure 16 Env regions covered by elicited Env cell-mediated immune responses.

Identified epitopes were mapped to the appropriate location within Env for each vaccine group as labeled on the left. Epitopes are shown as boxes of the length covered and on the right the percentage of Env covered by these epitopes is shown. Con B epitopes are shown in black and Con C epitopes are shown in grey.

Table 4 Number of peptides identified per region of Env.

Con B ^a	C1	V1V2	C2	V3	C3	V4	C4	V5	C5	Ecto	Tm	Total
Con B ^b	5	3	4	1	3	0	2	2	1	5	9	35
Con C ^b	11	5	3	1	4	1	3	0	2	1	4	35
Total	16	8	7	2	7	1	5	2	3	6	13	70
Poly B ^a	C1	V1V2	C2	V3	C3	V4	C4	V5	C5	Ecto	Tm	Total
Con B ^b	5	1	3	0	1	0	1	1	1	3	12	28
Con C ^b	5	2	4	0	2	2	2	0	1	3	11	32
Total	10	3	7	0	3	2	3	1	2	6	23	60
PVO.4 ^a	C1	V1V2	C2	V3	C3	V4	C4	V5	C5	Ecto	Tm	Total
Con B ^b	0	0	0	0	0	0	0	0	0	0	1	1
Con C ^b	0	0	0	0	0	0	0	0	0	0	2	2
Total	0	0	0	0	0	0	0	0	0	0	3	3
Con C ^a	C1	V1V2	C2	V3	C3	V4	C4	V5	C5	Ecto	Tm	Total
Con B ^b	9	2	4	2	6	0	2	4	3	5	13	50
Con C ^b	3	1	3	1	4	2	2	0	3	2	2	23
Total	12	3	7	3	10	2	4	4	6	7	15	73
Poly C ^a	C1	V1V2	C2	V3	C3	V4	C4	V5	C5	Ecto	Tm	Total
Con B ^b	3	0	1	2	1	0	0	2	0	1	5	15
Con C ^b	3	0	2	1	1	0	1	1	1	2	5	17
Total	6	0	3	3	2	0	1	3	1	3	10	22
DU151 ^a	C1	V1V2	C2	V3	C3	V4	C4	V5	C5	Ecto	Tm	Total
Con B ^b	2	1	3	1	3	1	1	1	2	3	2	20
Con C ^b	2	3	6	1	3	0	2	3	1	3	8	32
Total	4	4	9	2	6	1	3	4	3	6	10	52

^a vaccine group.

^b Env peptide set, number represents the number of peptides identified per region of Envelope

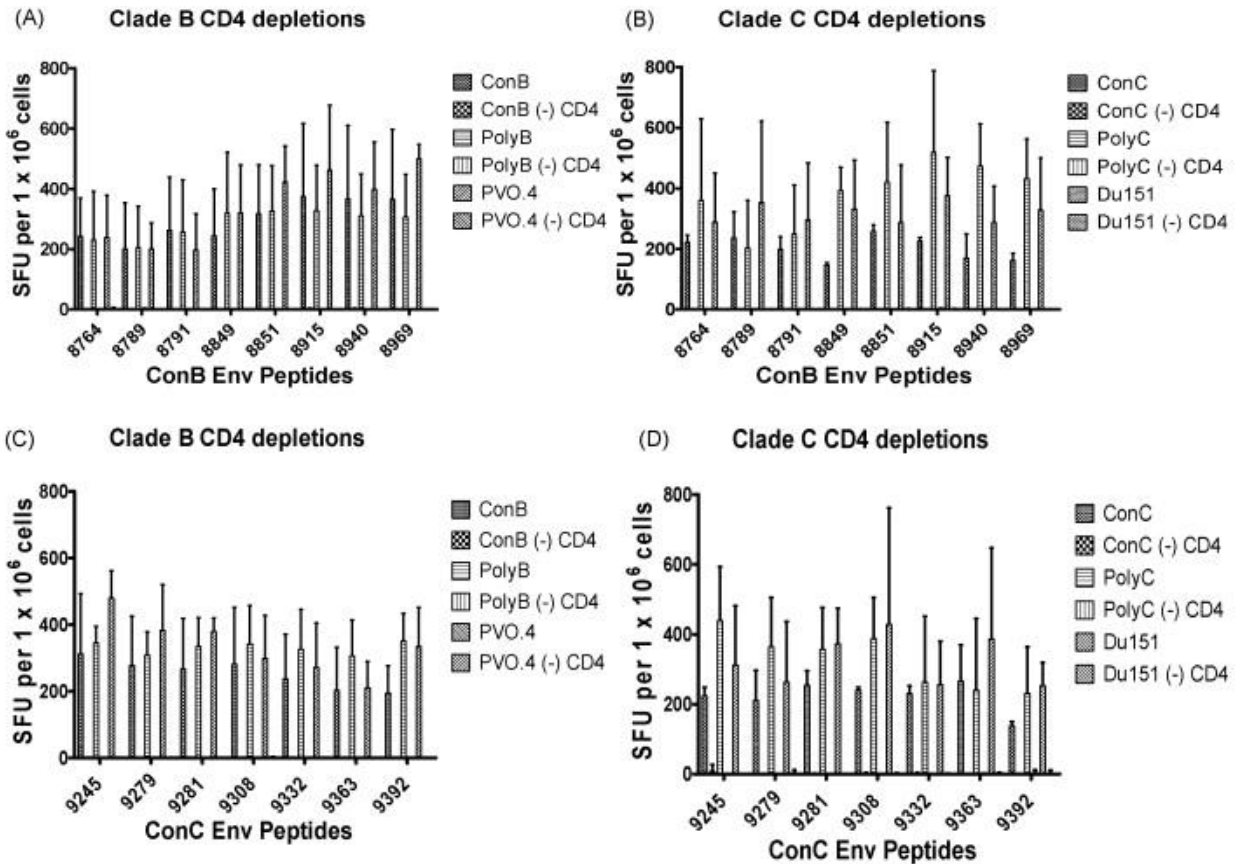


Figure 17 Identified epitopes require CD4⁺ T lymphocytes.

The most common and reactive epitopes from Con B and Con C envelopes were used to determine if the identified epitopes were CD4 or CD8⁺ T cell Responses. Splenocytes were isolated and stimulated with individual peptides (8 Con B and 7 Con C) either with or without CD4 cell depletion. Responses are represented as SFU per million cells. The values of each column are the mean \pm standard deviation.

Table 5 Percent sequence homology with Env peptide sets.

Env sequences/Env peptides	Con B Env peptides	Con C Env peptides
Consensus B	99.0	84.5
PVO.4	90.0	81.4
AC10.0.28	89.6	81.2
RHPA4259.7	91.1	82.9
SC422661.8	91.4	82.2
Consensus C	84.0	97.5
DU151	82.5	93.0
CHN19	81.5	88.4
ZM214	85.3	89.3

4.5 DISCUSSION

The intrinsic diversity among circulating populations of HIV-1 in various geographical locations is one of the greatest challenges for developing an effective AIDS vaccine. There is a great need to develop vaccines that can elicit enduring protective immunity to variant HIV-1 strains. While variation is observed in all of the viral proteins, the greatest diversity is localized to the viral envelope glycoproteins, evidently reflecting the predominant role of these proteins in eliciting host immune recognition. In this study, we examined the effectiveness of two vaccine strategies (polyvalent and consensus) for eliciting broadly reactive immune responses against Envs from diverse clade B and C isolates. Virus-like particles were used to deliver these envelopes in a native, trimeric structure [255] to a mucosal surface that effectively elicited cellular immune responses. Each VLP vaccine expressed similar amounts of envelope on the particle surface (Fig. 12) and therefore differences in the elicited immune responses were due to the particular envelope and not the amount of Env antigen delivered.

All VLP vaccines were highly immunogenic with each VLP vaccine eliciting neutralizing antibodies against a panel of viral isolates. However, viral Gag_{p24} particles elicited similar neutralizing titers indicating that a majority of the neutralization activity was most likely directed to cellular proteins embedded in the viral membrane and were not Env-specific. Similar results were also observed following vaccination with VLPs or inactivated virus in rabbits and guinea pigs (our unpublished observations and [256]). The VLPs used in this study were produced in non-human primate cells and therefore the particle membrane was coated with host cellular proteins that are foreign to the mouse immune system and thereby elicit strong immune responses. While this may be arguably beneficial for a VLP vaccine, it limited the ability to determine the effectiveness of a polyvalent or consensus Env vaccine strategy to elicit broadly reactive neutralizing antibodies. Therefore, future studies in primates, possibly using DNA or viral vectors expressing the Envs from a VLP *in vivo*, will be necessary to answer these questions.

Each VLP vaccine elicited strong anti-Env cell-mediated immune responses as detected by both the Con B and Con C Env peptide sets. These sets were chosen since they represented all of clade B and C viruses instead of any single isolate. The Con B sequence used in the peptide set was ~90% identical to the four Envs sequences used in the Poly B Env VLP vaccine (Table 5) and they were ~82% identical to the Con C peptide set. Similar results were observed with the clade C isolates to the Con C peptide set. Both consensus and polyvalent Env vaccine strategies elicited cell-mediated immune responses throughout all regions of Env. However, the number of specific epitopes elicited by Con B and Poly B Env VLPs was trended towards higher responses than the monovalent clade B Env VLP (Fig. 15). No matter how potent a response to a particular epitope in envelope is, it is not advantageous if the vaccine responses are focused on

epitopes that are retained only in a small percentage of HIV-1 isolates [232, 236]. Focusing vaccine responses on epitopes that have escaped and are rare in the current population of virus isolates may be a disadvantage for an effective vaccine. Con B and Poly B Env VLPs elicited responses that recognized 70 and 60 different peptides and covered 48.5% and 44% of the envelope, respectfully. The monovalent clade B Env VLP vaccine induced recognition of only three peptides and covered 2.55% of the envelope epitopes. In contrast, the monovalent DU151 VLP elicited cell-mediated immune responses as efficiently as Con C or Poly C Env VLPs against the Con C peptides. Interestingly, the Con B and Poly B Env VLP vaccines had greater or equal coverage of the Con C envelope than any of the clade C vaccines tested (Fig. 6). This is likely due to the higher reactivity observed with the clade B vaccines than the clade C vaccines. However, the Con C Env VLP elicited more cross-reactive responses that covered more clade B epitopes than either Poly C or monovalent clade C Env VLP vaccines.

We were concerned that using consensus peptide sets to compare consensus to polyvalent Env vaccine strategies would bias our results towards a consensus Env vaccine strategy. These peptide sets were chosen because they were readily available from the NIH AIDS Reference and Reagent Program. Interestingly, the Con B peptide set was not more likely to detect specific epitopes elicited by a Con B Env antigen compared to a mixture of Envs. In addition, similar results were observed using a peptide set derived from the clade B isolate, SF162 (data not shown). This was not surprising, since the SF162 had ~94% identical amino acids as the Con B sequence.

These VLP vaccines were administered intranasally to elicit mucosal immune responses. Intranasal immunization with VLPs induced both systemic and mucosal immunity. Mucosal infection serves as the major route of HIV infections in the world [257]. While circulating virus

and virus-specific immune responses are detected in the periphery shortly after infection, virus-specific immune responses at mucosal sites are critical for the control of infection in many individuals exposed to HIV-1 [258-260]. One advantage of using VLPs, compared to the low or undetectable level of immune responses elicited by soluble antigens delivered intranasally [219], is the administration of these VLPs, with CpG oligodeoxynucleotides as an adjuvant, enhances both systemic and mucosal immune responses [261-266]. This is most likely due to phagocytosis of the VLP immunogen by microfold epithelial cells (M cells) in the nasal lumen that leads to direct deposition of antigen to the nasal associated lymphoid tissue (NALT) via M cell transcytosis [267]. This process results in the induction of strong local (NALT), as well as distant immune responses in both peripheral and mucosal immune compartments [268].

Interestingly, there were differences in the regions of Env recognized by mucosal T cells using these vaccines compared to peripheral T cells (Fig. 14). All the vaccines elicited responses against V3 and gp41 regions of Env. However, the Con B and Con C Env VLPs elicited a broader recognition of Env regions in the mucosa than the monovalent or polyvalent Env VLP vaccines. The elicitation of a broader mucosal cell-mediated immune response by the consensus Envs may allow for recognition of a larger number of isolates at the site of infection. Future studies in non-human primates will be needed to determine if these VLP vaccines, administered intranasally, elicit vaginal mucosal immune responses. Intranasal immunization directly stimulates the vaginal mucosal immune system to elicit high titer responses [269-271] and therefore, VLP administration may be an effective strategy to elicit genital mucosal responses.

The aim of this study was to evaluate the breadth of coverage generated by Consensus and Polyvalent virus-like particle vaccines. The breadth of coverage was evaluated using consensus B and C envelope peptide sets. All vaccines were able to generate equal levels of

antibody and cellular responses systemically as well as mucosally. The qualities of these responses were not equal. Both the ConB and ConC VLP vaccines generated responses that recognized a greater number of Env epitopes thereby creating a greater coverage of Env than either polyvalent vaccine. These results indicate that a Consensus VLP vaccine would likely provide the greatest protection against a wide range of isolates as compared to a polyvalent vaccine.

5.0 SPECIFIC AIM 3

Antibody mediated prevention of heterologous vaginal SHIV_{SF162p4} infection through vaccination with a Polyvalent clade B virus-like particle vaccine

Sean P. McBurney¹, Meredith Hunter³, Preston A. Marx³,
Donald N. Forthal⁴ and Ted M. Ross^{1,2,*}

¹Center for Vaccine Research and ²Department of Microbiology and Molecular Genetics
University of Pittsburgh, Pittsburgh, Pennsylvania, USA

³Division of Microbiology, Tulane National Primate Research Center,
Tulane University Health Sciences Center, Covington, Louisiana, USA

⁴Division of Infectious Diseases, Department of Medicine,
University of California, Irvine School of Medicine, Irvine, California, USA

5.1 FOREWORD

This aim was completed in collaboration with the Preston Marx at the Tulane National Primate Center. All NHP manipulations and sample collections were overseen by The Marx Lab manager, Meredith Hunter. ADCVI activity was measured by Donald N. Forthal. All other immune responses were measured by Sean P. McBurney. The authors would also like to thank Hermancia Eugene and Dilhari DeAlmeida for their contribution to purifying VLP for this study.

5.2 ABSTRACT

The vast diversity of HIV-1 infections has greatly impeded the development of a successful HIV-1/AIDS vaccine. Previous vaccine work has demonstrated limited levels of protection against SHIV/SIV infection, but protection was only observed when the challenge virus was directly matched to the vaccine strain. As it is likely impossible to directly match the vaccine strain to all infecting strains in nature, it is necessary to develop an HIV-1 vaccine that can protect against a heterologous viral challenge. In this study we investigated the ability of polyvalent and consensus vaccines to protect against a heterologous clade B challenge. Rhesus macaques were vaccinated with ConB or PolyB virus-like particle vaccines. All vaccines were highly immunogenic with high titers of antibody found in all vaccinated groups against SIV Gag. Antibody responses were also observed against a diverse panel of clade B envelopes. Following vaccination NHPs were challenged via the vaginal route with SHIV_{SF162p4}. The PolyB vaccine induced a 66.7% reduction in the rate of infection as well as causing a two log₁₀ reduction in viral burden if infection was not blocked. ConB vaccination had no effect on either the infection

rate or viral burden. These results indicate that a polyvalent clade matched vaccine is better able to protect against a heterologous challenge as compared to a consensus vaccine.

5.3 INTRODUCTION

It is estimated that 33 million people worldwide are currently living with HIV-1 with 2.7 million people becoming newly infected in 2008 highlighting the need for a preventative vaccine [272]. One of the greatest struggles against developing an HIV-1 vaccine is the large diversity of viral isolates with differences in envelope sequences, which differ by as much as 10% within a given clade and 35% across clades [273]. Previous vaccine studies in non-human primates demonstrated sterilizing immunity, but protection was only observed when the vaccine was exactly matched to the challenge strain [223-224, 226, 274-275]. An effective HIV/AIDS vaccine will need to protect against heterologous viral challenges.

Various strategies have been investigated to address the issue of Env diversity (for review see [276]). Polyvalent vaccines have been found to be an effective strategy in the protection against a number of infections including Pneumococcus, Influenza, and Polio [105]. Polyvalent vaccines are typically composed of multiple copies of a given target(s), thereby increasing the diversity of the epitopes presented to the immune system. If the diversity of the epitopes is large enough within the polyvalent vaccine, it can present one or more epitopes present in any given isolate. Polyvalent HIV/AIDS vaccines do increase the breadth and strength of both cellular and humoral immune responses compared to monovalent vaccines [107-108, 114-116, 118, 227-228, 277-278].

Another strategy to address the issues of Env diversity is the construction of envelope antigens based upon a consensus sequence derived from numerous HIV-1 isolates. These vaccines utilize a consensus sequence that has been artificially generated to represent the most common amino acid at each position of a given target from a collection of sequences. The goal of this strategy is to minimize the genetic difference between the vaccine strain and any given primary isolate. Previous studies have indicated that consensus Env proteins are functional and highly immunogenic [84-85, 90-94, 277]. Consensus vaccines can induce a broader immune response as compared to a primary isolate [277].

The first aim of this study was to compare the ability of a consensus clade B (ConB) and a polyvalent clade B (PolyB) Env vaccine to develop a broadly reactive immune response in a non-human primate model. Both vaccines were delivered on the surface of a virus-like particle to facilitate the presentation of envelope in its native conformation. The second aim was to determine the ability of a consensus and polyvalent vaccine to protect against a SHIV challenge. Following vaccination, all non-human primates (NHPs) were challenged with a SHIV_{SF162p4} via the intravaginal route. SHIV_{SF162p4} was heterologous to both the ConB and PolyB vaccines thus better representing a potential transmission event. The vaginal route was chosen as this is the most common transmission route worldwide [279]. This is the first study to directly compare the breadth of immunity generated by a consensus and polyvalent vaccine in a non-human primate model.

5.4 MATERIAL AND METHODS

5.4.1 DNA Plasmids

The pTR600 vaccine plasmid [237] and the HIV-1 VLP expressing plasmid, have been previously described [238]. Briefly, the pHIV-wtVLP_{ADA} plasmid encodes for the following gene sequences: HIV-1_{BH10} *gag-pol* (pHIV_{BH10} nt 112–3626) (accession number M1564) and HIV-1_{ADA} *vpu*, *env*, *rev*, *tat* (nt 5101–8159). Safety mutations were engineered into Gag to prevent viral RNA packaging [239-240] and RT to prevent reverse transcriptase and RNase H activity (pHIV-VLP_{ADA}) [23, 241-242]. A codon optimized SIV_{Mac239} p55 Gag gene (generous gift from Dr. Andrea A. Gambotto) was cloned into pTR600 to generate the SIV Gag VLP. Each VLP was expressed from a cytomegalovirus immediate-early promoter (CMV-IE) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription.

Consensus VLPs were constructed by substituting ADA *env* with the consensus *env* sequences from the consensus clade B envelopes (LANL database). These sequences represent the most common amino acids found at each location within the Env gene from over 200 isolates for each clade. The primary isolate VLPs were constructed by substituting ADA *env* with the primary *env* sequence. These isolates were obtained from the AIDS Reagent and Reference Program. These isolates were chosen as they were isolated from diverse geographic locations during the acute phase of HIV infection[183, 253]. All VLPs have been previously shown to incorporate similar amounts of envelope [277].

Each plasmid was amplified in *Escherichia coli* strain-DH5 alpha, purified using anion-exchange resin columns (Qiagen, Valencia, CA), and stored at -20°C in dH₂O. Plasmids were

verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at a wavelength of 260 and 280 nm.

5.4.2 Purification of virus-like particles

Supernatants from COS cells, transiently transfected with plasmid expressing Gag or VLPs were purified via ultracentrifugation (100,000 X g through 20% glycerol, weight per volume) for 4 h at 4°C as previously described [255]. The pellets were subsequently resuspended in PBS and stored at -20°C until use. Protein concentration was determined by Micro BCA™ Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA).

5.4.3 MHC haplotype determination

MHC haplotyping was completed by the MHC Typing core at the University of Wisconsin AIDS Vaccine research laboratory (Madison, WI, USA). Briefly 2 ml of EDTA anti-coagulated blood was collected and sent shipped overnight at 4°C. MHC genotyping was completed using PCR-SSP typing for A01, A02, A08, A11, B01, B03, B04, B08, and B17 genotypes.

5.4.4 Immunization of Rhesus macaques

Rhesus macaques, *Macaca mulatta*, were anesthetized with ketamine (10mg/kg). Vaccinations were completed at 8 week intervals over 32 weeks. The first two vaccinations were given as DNA vaccinations. All groups received 1 mg each of DNA encoding the HIV VLP containing Env and SIV Gag VLP in 1ml saline via an IM injection. All groups received 4 µg of HIV VLP

and SIV VLP plasmids via gene gun (Bio-Rad, Hercules, CA, USA) over the inguinal lymph nodes. The third and fourth vaccinations were administered as 250 µg each of purified HIV VLP and SIV VLP with CpGs, 125 µg. The vaccines were split with 125 µg given via an intramuscular injection in the quadriceps and the second 125 µg given via the intranasal route. Each CpG ODN: K3- ATCGACTCTCGAGCGTTCTC, D35- GGTGCATCGATGCAGGGGGG, K123- TCGTTCGTTCTC, D29- GGTGCACCGGTGCAGGGGGG, D19- GGTGCATCGATGCAGGGGGG [280] was synthesized and purified by high-pressure liquid chromatography (Sigma-Genosys, The Woodlands, TX, USA). The CpG ODNs were resuspended in sterile dH₂O (2 µg/µl) and stored at -20°C.

5.4.5 Intravaginal Challenge

All animals were challenged intravaginally with SHIV_{SF162p4} which was obtained from Drs. Nancy Miller (DAIDS, NIAID) and Dr. Ranajit Pal (ABL, Kensington, MD, USA). Briefly all animals were anesthetized using an intramuscular injection of either Ketamine or Telazol before virus exposures. All virus exposures were a 1 ml dose of 640 TCID₅₀ with the animal in the supine position which was maintained for at least ten minutes. Viral exposures were completed on days 0, 3, 7, and 10.

5.4.6 Collection of Samples

Animals were anesthetized using an intramuscular injection of either Ketamine (10mg/kg) or Telazol (0.1ml/kg). Blood was collected from the femoral vein using the Sarstedt Monovette

blood collection system (Sarstedt, Inc., Newton, NC, USA). PBMCs were collected and purified using ACCUSPIN™ System-Histopaque®-1077 tubes according to the manufactures instructions (Sigma-Aldrich, St. Louis, MO, USA). Vaginal washes were collected following anesthetization with Telazol. The vaginal mucosa was washed with 5 ml of sterile PBS. Samples were collected and centrifuged to separate cells and supernatants at 3000 rpm for 10 min. Cells and supernatant were taken separately and stored at -80°C until used.

5.4.7 Viral load Determination

Viral RNA (vRNA) levels were determined by Siemens Diagnostics Clinical Laboratory using the SIV RNA 4.0 bDNA Assay (Siemens Diagnostics, Berkeley, CA, USA). Plasma samples, 0.5 ml, were collected as stated above and sent to Siemens for processing. Briefly Plasma was concentrated via centrifugation. The SIV genomic RNA is then captured to a microwell by probes targeted to the *pol* gene. The pre-amplifier probes are then added binding to the vRNA. Amplifier probes are then added forming a branched DNA (bDNA) complex. Substrate is added resulting in light emission which is directly proportional to the amount of vRNA present in the sample. A standard curve is used to determine the concentration of vRNA per sample.

5.4.8 CD4/CD8 Cell count

EDTA-treated whole blood was stained using CD3, CD4 and CD8 monoclonal antibodies and analyzed using a BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA). Cell counts were determined using the BD Truecount tubes according to the manufacture's instructions (BD Biosciences, San Jose, CA, USA).

5.4.9 CD8⁺ lymphocyte depletion

Rhesus macaques were treated with the antibody cM-T807 (Nonhuman Primate Reagent Resource, Boston, MA, USA). This treatment was started at day 160 post-infection. Antibody was given on day 0 post-infection at a dose of 10mg/kg given subcutaneously. Additional doses were given at days, 3, 7, and 10 at a dose of 5 mg/kg given via the intravenous route. Intravenous treatments were given as a slow bolus over 10-20 minutes. CD8 depletion was confirmed by flow-cytometry.

5.4.10 Antibody Responses to VLP immunization

Serum and mucosal wash samples were individually collected and tested for antibody (IgG) responses to SIV Gag and multiple gp120 isolates by ELISA. For the Env ELISAs each well of a 96-well plate was pretreated with 5 µg of concanavalin A (Con A) for 1 hr at 25°C. For all ELISAs each well of a 96-well plate was coated with 50 ng per well of the respective protein (4°C for 16 hr). Plates were blocked (25°C for 2 hr) with PBS containing Tween 20 (0.05%) and nonfat dry milk (5%) and then incubated with serial dilutions of each sample (25°C for 2 hr). Following thorough washing in PBS-Tween 20 (0.05%), samples were incubated (25°C for 1 hr) with biotinylated goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) diluted 1:5000 in PBS-Tween 20 (0.05%) and nonfat dry milk (5%). The unbound antibody was removed, and the wells were washed. Samples were incubated with TMB substrate (1 hr), and the colorimetric change was measured as the optical density (O.D., 405nm) by a spectrophotometer (BioTek Instruments, Winooski, VT, USA). The O.D. value of naïve sera

was subtracted from the samples using antisera from vaccinated mice. Results were recorded as the arithmetic mean \pm the standard deviation.

5.4.11 Neutralization Assay

Antisera were tested for the ability to neutralize virus infection *in vitro* using TZM-BL cells indicator cells [251-252]. These cells express human CD4 (hCD4), human CCR5 (hCCR5), human CXCR4 (hCXCR4), and a luciferase reporter driven by the HIV-1 LTR. TZM-BL cells were cultured in cDMEM with 10% fetal calf serum (10%) (Atlanta Biologicals, Atlanta, GA, USA). Infectivity was determined using serial dilutions of antisera with cells in complete, non-selective media in the presence of DEAE dextran (20 μ g/ml) (25°C for 1hr). Cell lysates were harvested in lysis buffer (25mM Tris phosphate, pH=7.8, 2mM DTT, 2mM 1-2-diaminocyclohexane-N, N, N', N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) (48 h) and then clarified by centrifugation. Virus neutralization by antisera was determined by measuring the relative light units (RLU) using an Orion Microplate Luminometer (Berthold Detection Systems, Pforzheim, Germany).

5.4.12 ELISpot Assays

The number of anti-Gag and anti-Env specific nonhuman primate IFN- γ (mIFN- γ) secreting splenocytes was determined by enzyme-linked immunospot (ELISPOT) assay (R & D Systems, Minneapolis, MN, USA). Briefly, pre-coated anti-IFN- γ plates were incubated (25°C for 2 h) with cRPMI (200 μ l) and then were incubated with PBMCs (2.5×10^5 /well). PBMCs

were stimulated (48 h) with peptides (15mers overlapping by 11 amino acids) representing the SIV_{Mac239} Gag or SF162 Env proteins (NIH ARRRP). Control wells were stimulated with PMA(+) (50 ng)/ionomycin (500 ng) or were mock stimulated (-). Plates were washed with PBS-Tween (3X) and were incubated (37°C for 24 h; 5% CO₂) with biotinylated anti-mIFN- γ and incubated (4°C for 16 h). The plates were washed and incubated (25°C for 2 h) with streptavidin conjugated to alkaline phosphatase. Following extensive washing, cytokine/antibody complexes were incubated (25°C for 1 h) with stable BCIP/NBT chromagen. The plates were rinsed with dH₂O and air dried (25°C for 2 h). Spots were counted by an ImmunoSpot ELISpot reader (Cellular Technology Ltd., Cleveland, OH, USA).

5.4.13 Antibody-dependent cell mediated virus inhibition

Antibody-dependent cell-mediated viral inhibition (ADCVI) antibody activity was measured using methods similar to those described previously [149]. Briefly, target cells (CEM.NKR-CCR5 cells infected with SHIV_{SF162p4} virus for 48 h) were incubated with serum and with fresh human PBMC effector cells (effector/target ratio = 10:1). Seven days later, p27 from the supernatant was determined by ELISA (Zeptometrix Corporation, Buffalo, NY).

5.4.14 Statistical analysis

Anti-SIV Gag antibody responses, viral peak, viral burden and anti-SF162 Env antibody responses were compared pair wise using a one sided student's t test. The breadth of envelope binding was analyzed using a One-way ANOVA test along with Tukey's multiple comparisons secondary test.

5.5 RESULTS

5.5.1 Vaccines

The goal of this study was to enhance the breadth of immune responses directed against HIV-1 envelope. To achieve this goal, a mixture of wild-type (polyvalent) or consensus envelopes were used as immunogens on the surface of a virus-like particle (VLP). These vaccines were measured by determining their ability to elicit broadly reactive immune responses and to protect against a heterologous infection. A consensus clade B (ConB) and a polyvalent B (PolyB) VLP vaccine were based on an identical virus-like particle background with the only difference being the envelopes expressed on the surface (Table 6). As previously described [277], the consensus Env sequences were generated by selecting the most common amino acid at each position of a full-length Env alignment derived from 200 subtype B viruses deposited in the Los Alamos HIV Sequence Database. Each set of subtype sequences was supplemented with *env* sequences used in a standardized neutralizing panel [183]

The PolyB vaccine is composed of four separate VLPs with each expressing an individual Env isolate. These isolates were selected for their genetic, geographic, and transmission diversity. In addition, isolates were chosen only if they were isolated within a few weeks after transmission, since these early isolates may represent the majority of mucosally transmitted HIV isolates [281]. Vaccinations were administered systemically (intramuscular injection) and mucosally (intranasal delivery) to generate both mucosal and systemic immune

responses. Each non-human primate (NHP) was identified by a color-code within each given study group to facilitate easy comparisons between immune responses and infection outcome (Table 7).

Table 6 Virus-like Particle Vaccines

Vaccine	Isolate Name	Geographic Location of Patient	Mode of Transmission ^a	Length of Infection ^b
HIV Con B	Consensus B	---	---	---
HIV PolyB	PVO.4	Italy	M-M	4 weeks
	RHPA4259.7	USA	M-F	< 8 weeks
	SC422661.8	Trinidad	F-M	4 weeks
	AC10.0.29	USA	M-M	4 weeks

^a direction of sexual transmission: M-M male to male, M-F male to female, F-M female to male

^b length of infection is defined as time between last negative HIV-1 test and first positive HIV-1 test.

Table 7 Individual NHP Coding

Color	Group		
	Naïve	ConB	PolyB
BLACK	AH78	BK31	DV24
RED	CA23	BF28	AH69
BLUE	CC19	DJ34	T016
GREEN	BV03	BJ38	BT87

Each NHP per group was identified by a particular color to facilitate the following of pre-challenge immune responses against infection outcomes. Each NHP is was also given a unique code for further identification.

5.5.2 Immune Responses to VLP vaccination

Cellular immune responses were investigated against both SIV Gag_{p55} (mac239) and HIV-1 Env_{gp160} (SF162). Positive cellular responses were not detected either systemically (peripheral blood draw) or mucosally (intestinal lymph node biopsy) from any of the vaccine

groups (data not shown). However, both ConB and PolyB VLP vaccines elicited strong SIV Gag_{p55} antibody responses with a trend towards higher antibody responses induced by the PolyB VLP group compared to the ConB VLP vaccine group ($p=0.0676$) (Fig. 18). Limited SIV Gag antibody responses were also detected in vaginal wash samples (Fig. 18).

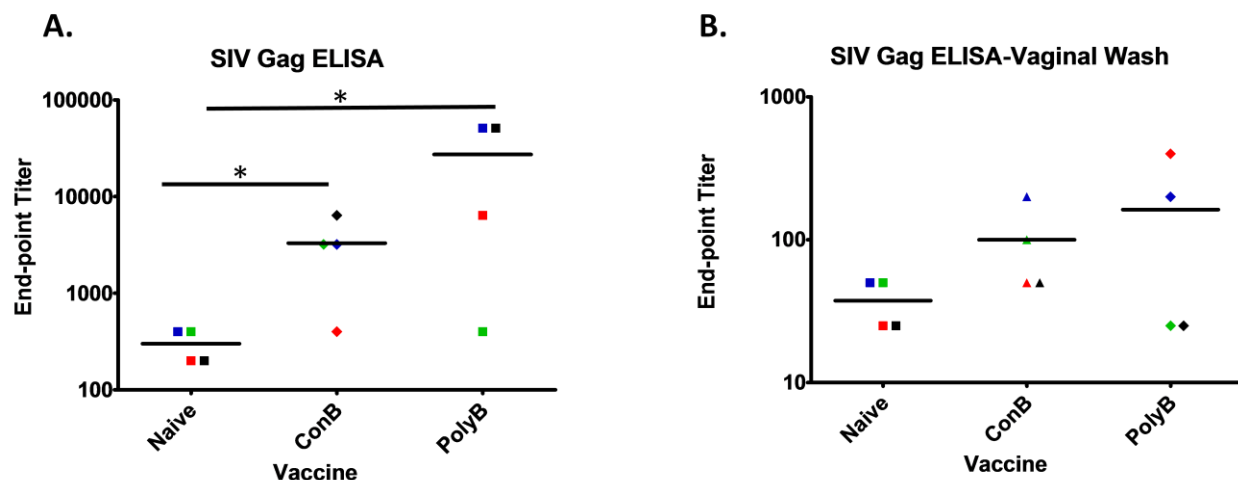


Figure 18 Systemic and mucosal antibody responses are generated by both vaccines.

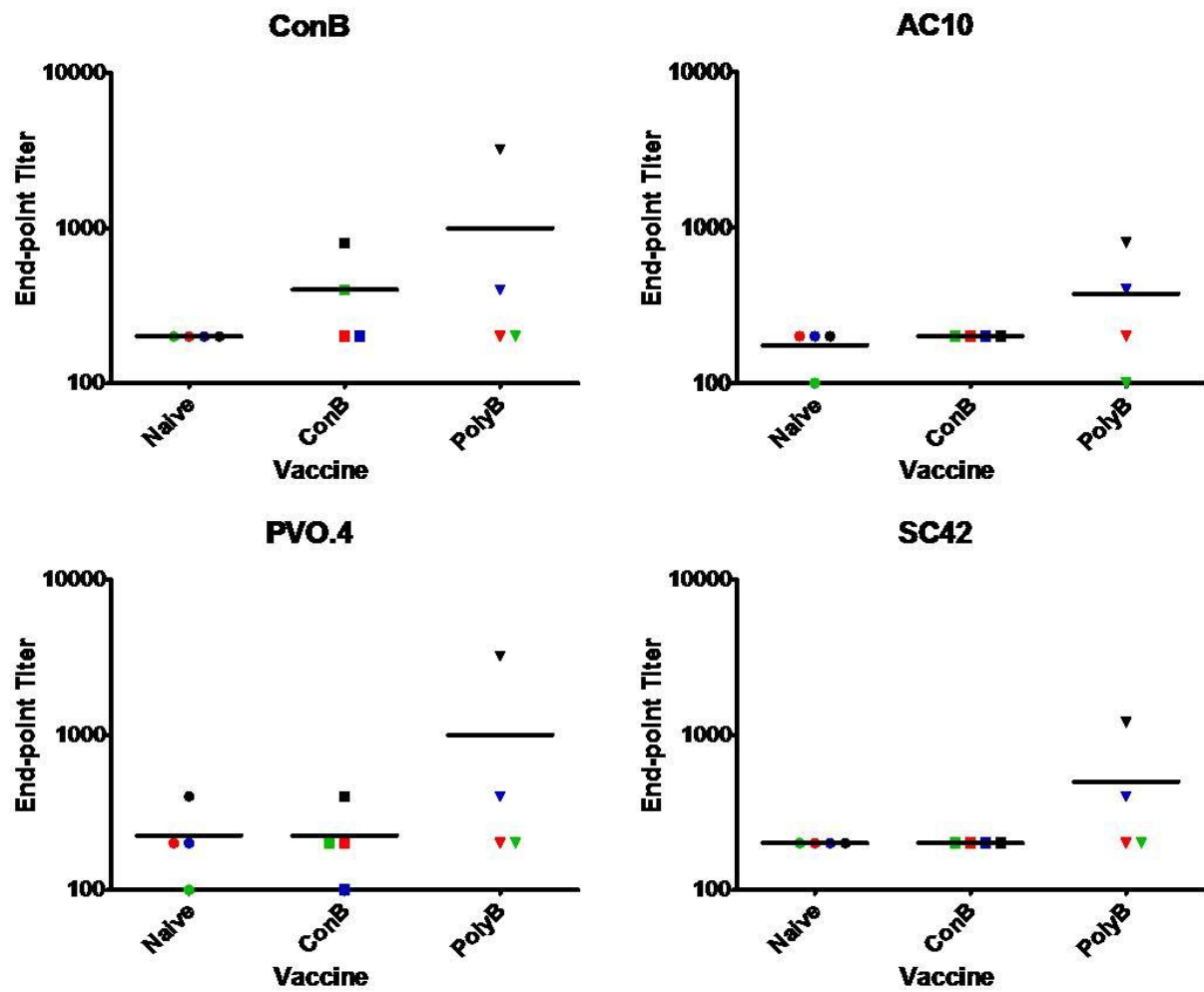
Serum samples and vaginal washes were taken two weeks following the completion of the vaccine regimen. Individual serum samples (A) and vaginal washes (B) were tested via serial dilution against SIV Gag p55. * Indicates p -value <0.05 , Statistics were completed using Student's T-test.

5.5.2.1 Breadth of anti-Env antibody responses

The primary goal of this study was to increase the breadth of viral isolates recognized. This was measured via Env binding ELISAs and Neutralization assay utilizing a panel of diverse Envelopes. First envelope antibody-binding titers against homologous Envelopes were determined (Fig. 19a). Against the ConB Env, only two of four NHPs from the ConB and the PolyB VLP group demonstrated recognition. Against the envelopes from the PolyB VLP

vaccine, The PolyB group recognized all isolates with at least two of four responding. Within the ConB VLP group, only PVO.4 was recognized by BK31. Next a panel of heterologous envelopes was tested (Fig. 19B). These envelopes were selected, as they were isolated early during infection and are hypothesized to be more representative of transmitting virus than late-stage isolates[183, 253]. Against these envelopes, only the PolyB VLP group demonstrated antibody binding to all isolates with at least one NHP recognizing all of the isolates tested. The ConB VLP group only demonstrated antibody binding against two of the envelopes, and each time only one NHP facilitated the recognition. The PolyB VLP group demonstrated a significantly greater breadth of anti-Env antibody responses than either the unvaccinated or ConB VLP groups (Tukey's multiple comparison test). Of particular interest was the response to the SF162 envelope, since this strain matches the challenge virus. Antiserum from one out of four NHPs from the ConB VLP group recognized the SF162 Env_{gp120}, while the serum from 2 out of 4 of the NHPs from the PolyB VLP group recognized the SF162 envelope (Fig. 20). Mucosal antibody responses, neutralizing antibodies and antibody-dependent cellular virus inhibition (ADCVI) were not detected against any of the envelopes tested (data not shown).

A.



B.

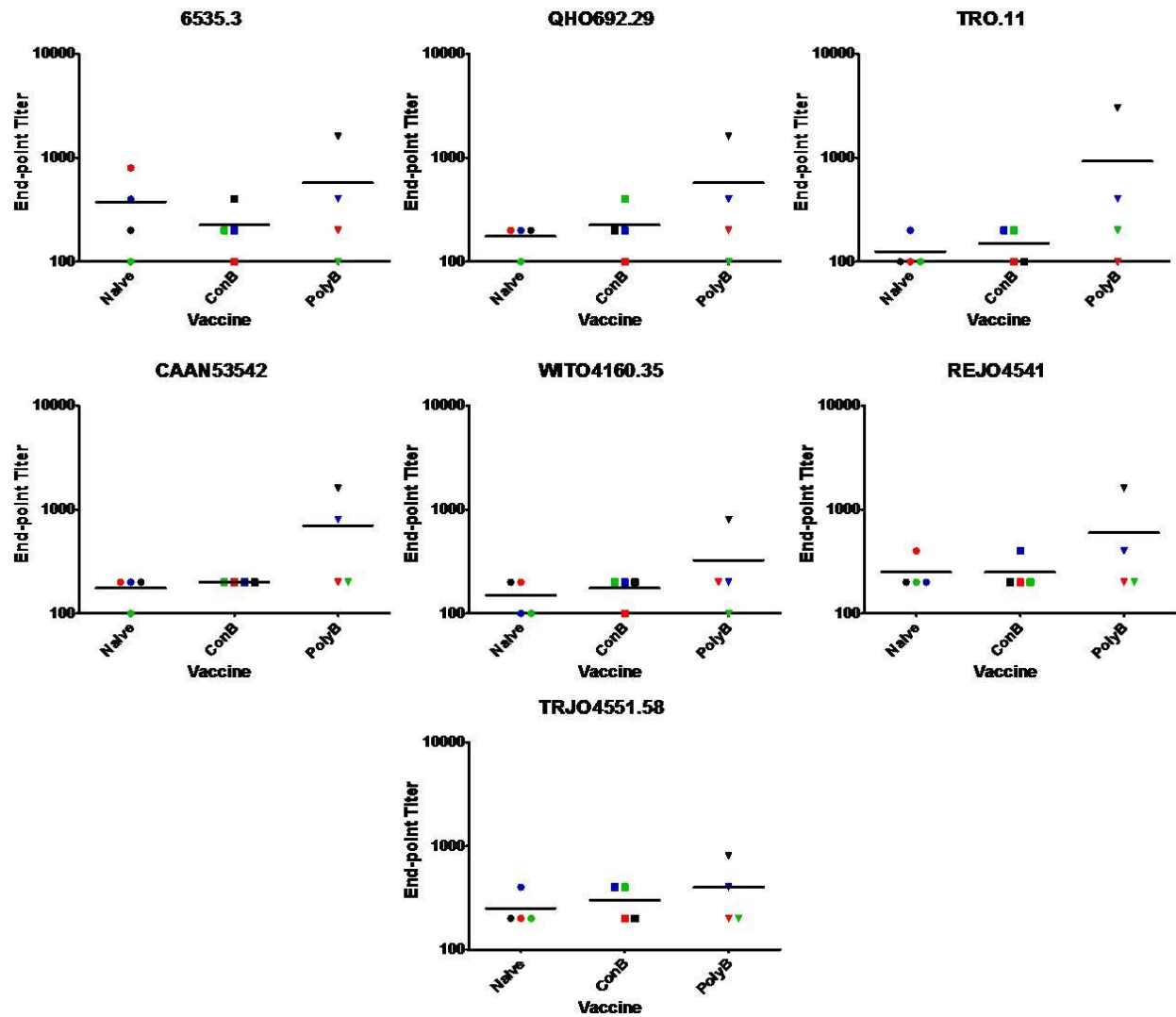


Figure 19 Breadth of pre-challenge anti-Env antibody responses.

Serum samples obtained two weeks following the final vaccination were tested via serial dilution against homologous (A) and heterologous (B) Env isolates. Results are represented as the average response from the four NHPs per group minus background responses observed in pre-vaccination samples.

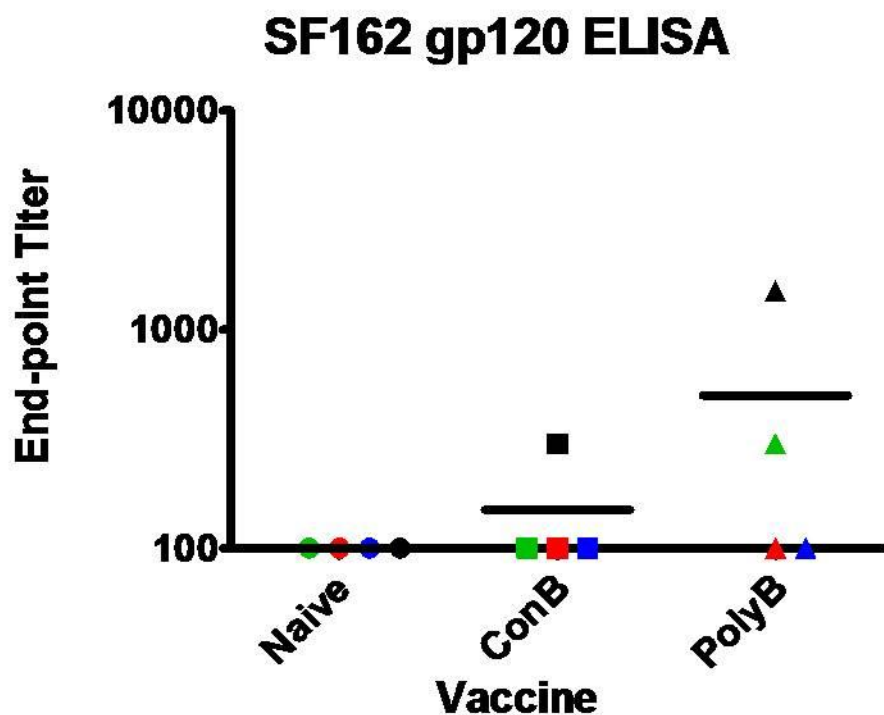


Figure 20 Anti-SF162 Envelope antibody responses.

Serum samples obtained two weeks following the final vaccination were tested via serial dilution against SF162 envelope.

5.5.3 Heterologous vaginal SHIV Challenge

Vaccinated NHPs (n=4) were challenged vaginally (4X) with SHIV_{SF162p4} (640 TCID₅₀) 8 weeks following the final vaccination [274, 282]. The envelope in this SHIV is not homologous to any of the envelopes used in the VLP vaccine formulation. The SHIV_{SF162p4} envelope has a ~86% amino acid homology with the four primary envelopes used in the PolyB VLP vaccine and a 91.3% homology with the ConB Env (Table 8). Seventy-five percent of unvaccinated control NHPs were infected with peak viral loads between 4.3×10^6 and 1.1×10^8 vRNA copies/ml (Fig. 21A). When normalized for initial day of infection, the peak viremia was observed at ~14 days post-infection. Normalization was necessary since infections could occur on either day 0, 3, 7,

or 10 depending on the viral exposure which established the infection. Similar results were observed with NHPs vaccinated with ConB VLPs who had peak viral titers between 5.4×10^5 and 2.6×10^7 vRNA copies/ml (Fig. 21C). In contrast, only one NHP (AH69) vaccinated with the PolyB VLP vaccine had detectable virus at any point during the infection period (Fig. 21E). The virus titer peaked in this animal at 1.4×10^5 vRNA copies/ml at day 14 post-infection, but returned to undetectable levels (>165 vRNA copies/ml) between days 54-70 post-infection. CD4⁺ lymphocyte levels were not significantly changed during the course of the infection (Fig 21B, D, & E). The total viral burden observed throughout the observation period was measured by determining the area-under-the-curve of the viral load (Fig. 22). The infection status of all non-infected NHPs did not correlate with any previously described protective MHC alleles [283-284] (Table 9).

Table 8 Envelope Sequence Homology

Vaccine	Envelope	Percent Homology to SF162 _{p4}
Consensus B (Con B)	ConB	91.3
Polyvalent B (Poly B)	PVO.4	85.6
	RHPA4259.7	86.2
	SC422661.8	86.4
	AC10.0.29	85.2

Table 9 MHC Alleles

	Animal	A01	A02	A08	A11	B01	B03	B04	B08	B17
Naïve	AH78	-	-	-	-	-	+	+	-	-
	CA23	-	-	-	-	+	-	-	-	-
	CC19	-	-	+	-	-	-	-	-	-
	BV03	-	+	-	-	-	-	-	-	-
ConB	BK31	-	+	+	-	+	-	-	-	-
	BF28	+	-	-	-	-	-	-	-	+
	DJ34	-	-	-	-	-	-	-	-	-
	BJ38	-	-	+	-	-	-	-	-	-
PolyB	DV24	-	-	-	+	+	-	-	-	-
	AH69	-	-	+	-	-	-	-	-	+
	T016	-	-	-	-	-	-	-	-	-
	BT87	-	-	-	-	+	-	-	-	-

Known protective MHC alleles are highlighted in red.

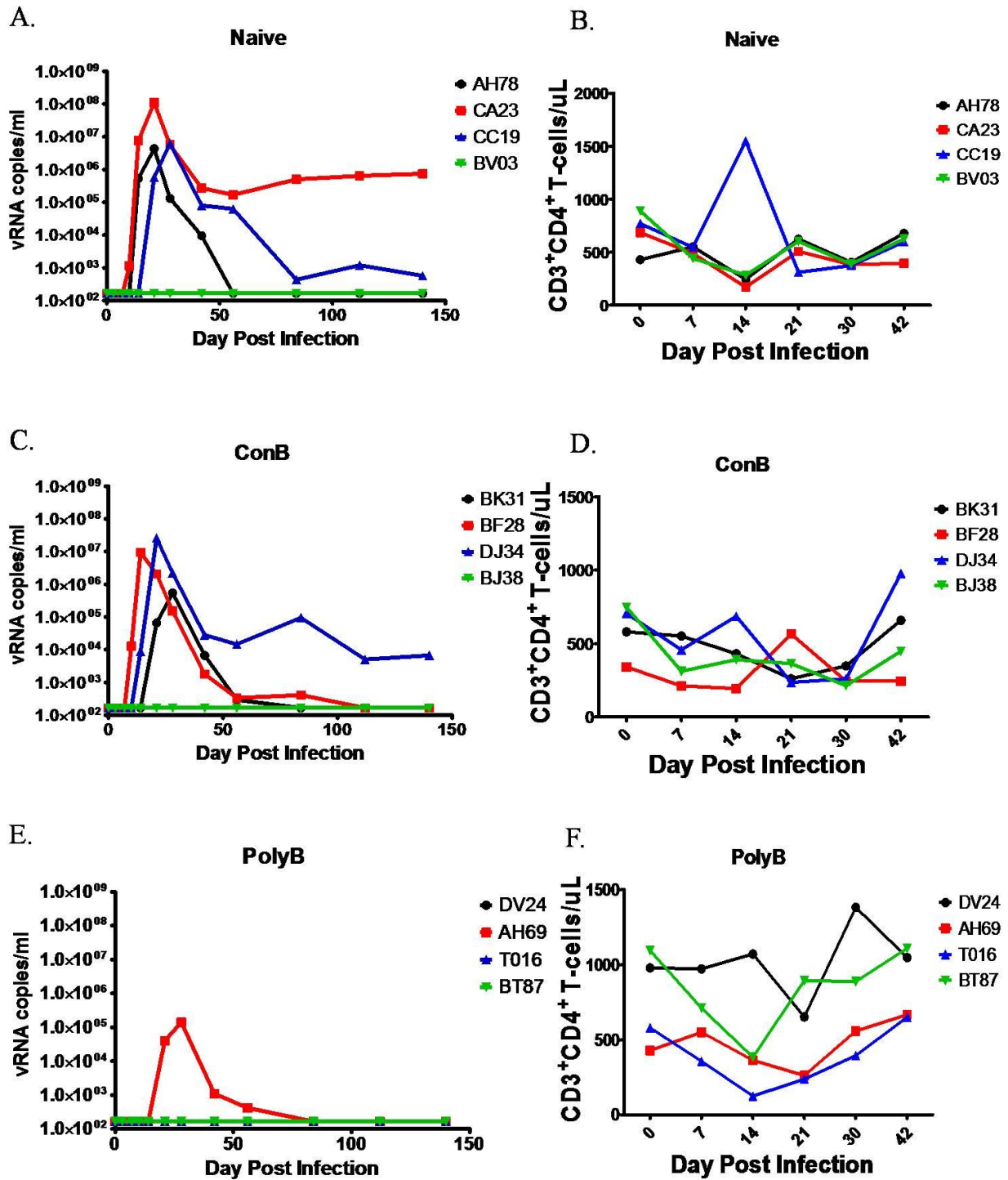


Figure 21 PolyB vaccination induced a reduction of viral load following heterologous challenge.

Viral RNA copies/ml in plasma samples and CD4⁺ lymphocyte counts: (A, B) Naïve, (C,D) ConB, and (D,E) PolyB

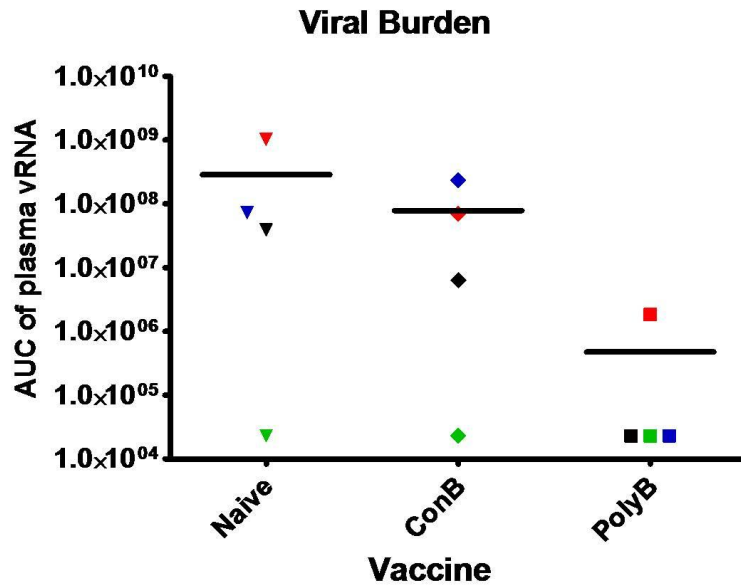


Figure 22 PolyB vaccination induced a reduction of viral burden following heterologous challenge.

Viral burden of infection measured by the area-under-the-curve of each individual viral load from day 0 to day 168 post-infection.

5.5.4 CD8⁺ lymphocyte depletion

All non-infected and infected NHPs who had undetectable virus at day 188 post-infection were depleted of CD8 T cells to release low viral titers in reservoir tissues. All NHPs were CD8-depleted via cM-T807 antibody treatment over 10 days. Complete depletion of CD8⁺ T cells in the blood was confirmed by flow cytometry in all treated NHPs (Fig. 23). All infected NHPs had a rebound virus detected in the blood by day 10 post-treatment (Fig. 23). All non-infected NHPs remained virus free through day 42 post-treatment, further confirming their non-infected status (Fig. 23).

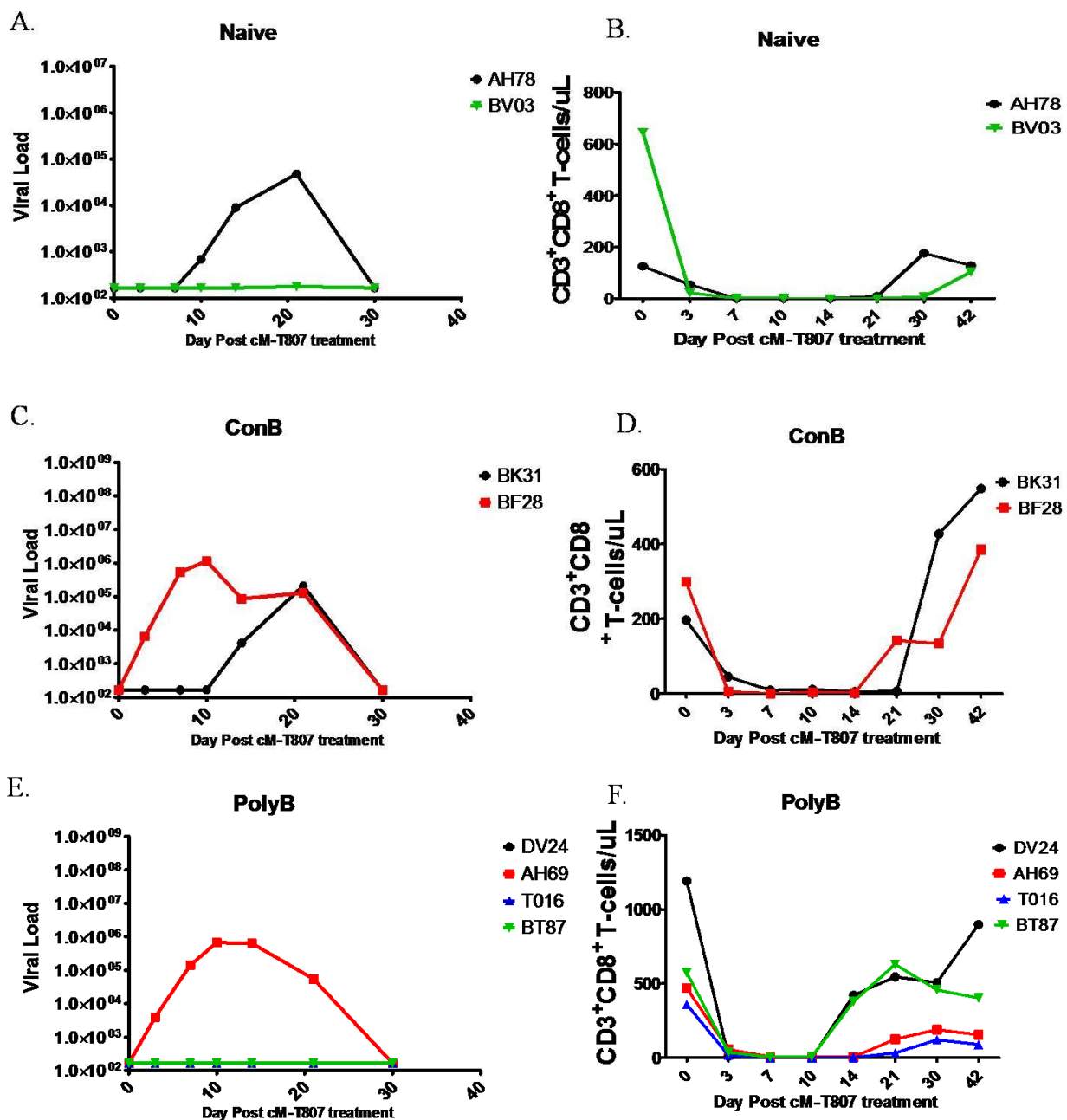


Figure 23 CD8⁺ lymphocyte depletion leads to expansion of viral load.

Viral RNA copies/ml in plasma samples and CD8⁺ lymphocyte counts: (A, B) Naïve, (C, D) ConB VLP vaccinated, and (E,F) PolyB VLP vaccinated.

5.5.5 Post-Challenge Immune Responses

Following challenge, there was a marked increase in antibodies to the SF162 Env in all infected NHPs, but not in NHPs without detectable virus. Throughout the post-challenge observation period, all non-infected NHPs had unchanged immune responses. As to not artificially decrease the levels of immune responses observed post-infection, all non-infected NHPs were not included in average antibody or neutralization titers. All NHPs were tested for all immune responses regardless of infection status. There was an initial increase in anti-Env antibody at day 21 that plateaued by day 54 post-infection and the level of anti-Env antibody achieved at day 84 post-infection correlated with viral burden (Fig. 24A & 24B). At day 42, low levels of neutralizing antibodies against the HIV-1 strain, SF162, were also detected. Neutralizing antibody levels continued to increase to day 56 and then were maintained through day 84 post-infection (Fig. 24B). ADCVI activity was also detected by day 84 post-infection (Fig. 24D). The kinetics of acquiring antibody responses were similar between all infected vaccinated and unvaccinated NHPs. The effect of vaccination on the breadth of anti-Env antibody responses post-infection was also investigated using a panel of envelopes. All groups demonstrated an increase in antibody binding breadth at day 84 post-infection as compared to pre-infection (Fig. 25 and 19). These responses were dependent on the establishment of infection with all anti-Env antibody responses being positively correlated to viral burden. While increased binding antibodies were observed, neutralizing antibodies were not detected to any envelopes tested excepted to SF162. There were no cellular responses detected against the SF162 envelope at any point during the challenge. In contrast, SIV Gag cellular responses were detected in all infected NHPs to similar levels (Fig. 26).

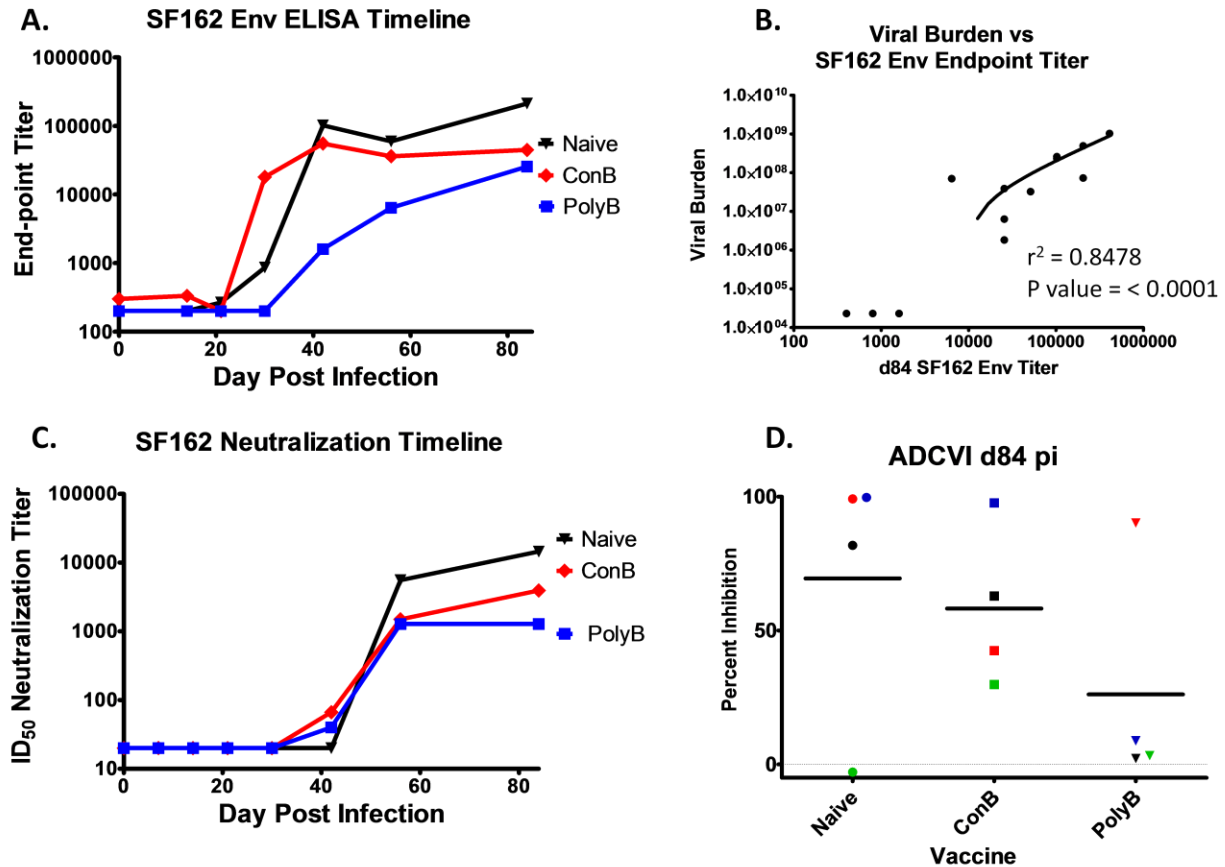


Figure 24 Viral Infection leads to the expansion of binding and neutralizing antibodies directed to SF162 Envelope.

Individual serum samples were tested for binding to SF162 envelope (A) as well as neutralization against SF162 pseudovirus (C). (B) Correlation of viral burden to SF162 end-point titer was completed by plotting day 84 end-point titer of infected NHPs against their respective viral burdens. Linear analysis was completed to determine line of best fit. (D) ADCVI activity against SHIV_{SF162p4} at day 84 post-infection.

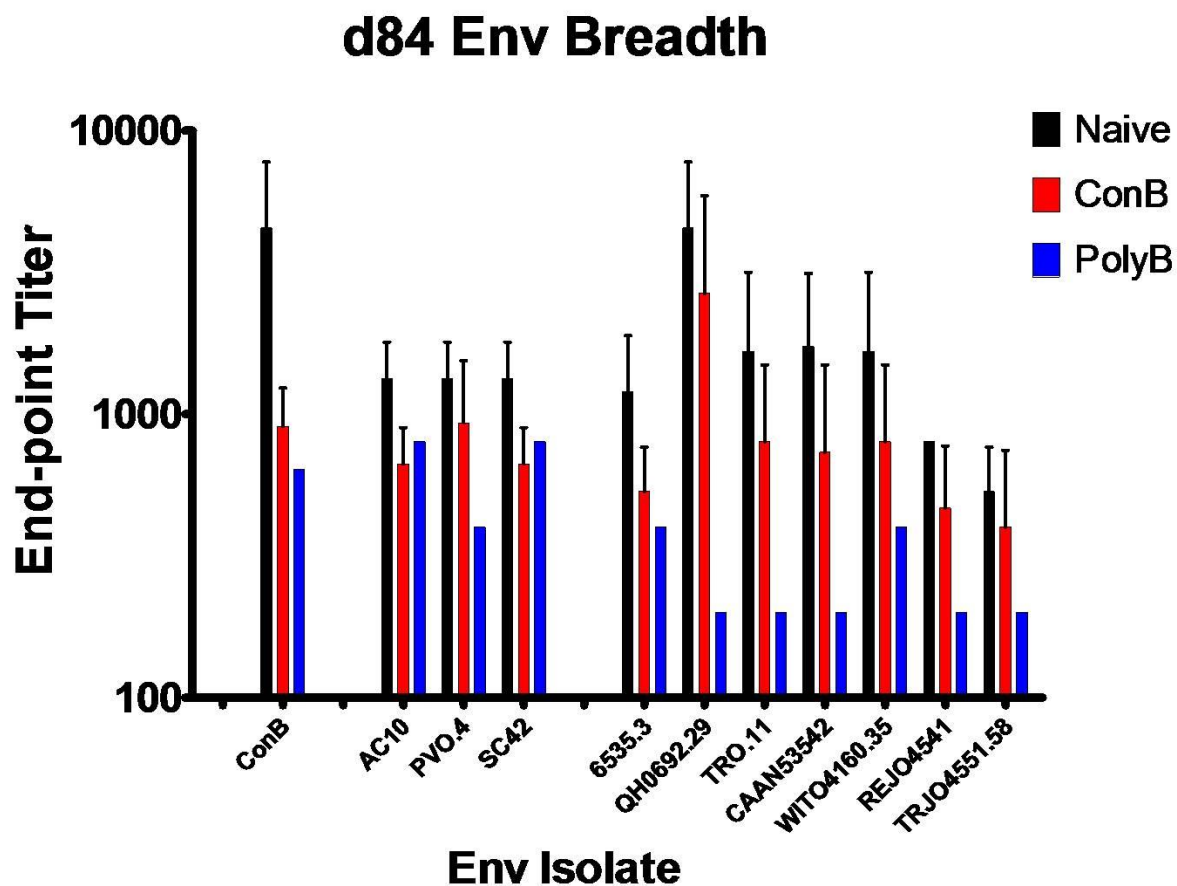


Figure 25 Post-Infection breadth of anti-Env antibody responses.

Serum samples obtained at day 84 post-infection were tested via serial dilution against Envs homologous and heterologous to the vaccines tested. Results are represented as the average response from only the NHPs who were infected. All non-infected NHPs were not included as their immune responses remained unchanged post-challenge.

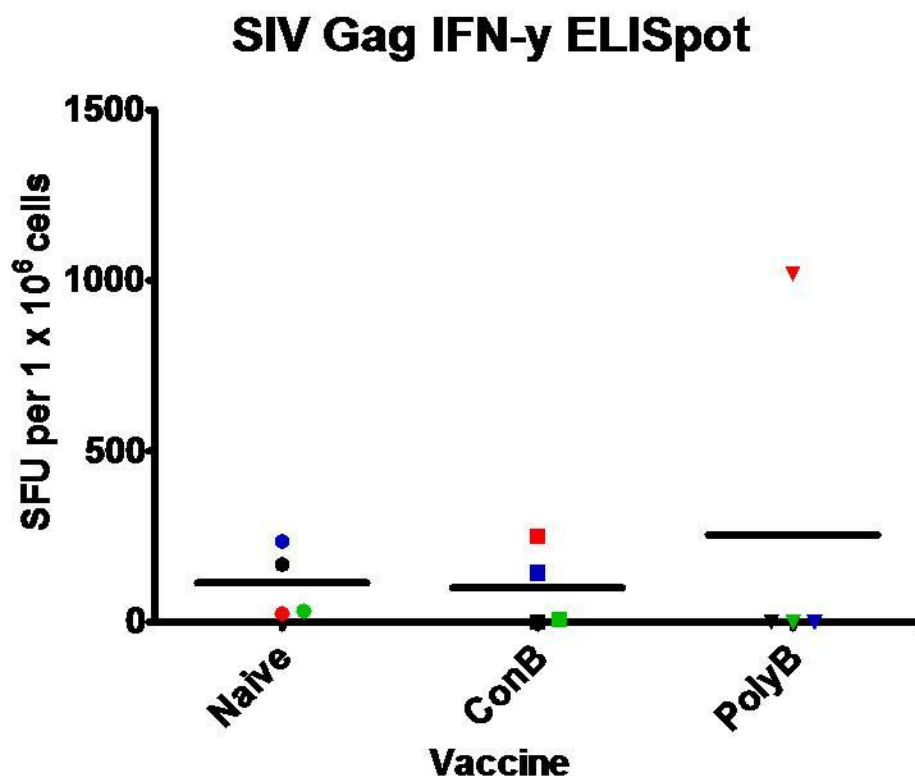


Figure 26 Infection leads to the development of anti-SIV Gag cellular Responses.

PBMCs were tested against an SIV_{Mac239} Gag peptide pools at day 56 post-infection. Results are shown as individual responses with the average response per group shown.

5.6 DISCUSSION

The vast diversity of HIV-1 particularly within *env* has greatly impeded the development of an HIV-1 vaccine, and it appears necessary to develop a vaccine that addresses this sequence diversity. In this study, we directly compared two strategies for developing broadly reactive vaccines: consensus and polyvalent. These vaccines were clade B specific with the intent to develop a vaccine that can protect against inter-clade isolates and then expand that strategy to address all of group M. Virus-like particles (VLPs) were used as the delivery mechanism for the

ConB and PolyB envelopes. VLPs present envelopes in their native, trimeric structure and also facilitate immune responses to the Gag core [255]. These VLPs incorporate similar amounts of envelope on their surface so any differences in the immune responses generated are due to immunogenic differences and not antigen dosage [277].

Both vaccines were immunogenic with the majority of recipients developing high antibody titers against SIV Gag with both ConB VLP and PolyB VLP vaccines eliciting immune responses statistically above naïve levels (Fig. 18). The PolyB VLP vaccinated NHPs also trended to have stronger anti-Gag antibody responses than ConB VLP vaccinees ($p=0.0676$) (Fig. 18). Interestingly these results indicate that the envelopes on the surface of the VLP affect the immunogenicity of the Gag core as both the ConB VLP and PolyB VLP vaccines were identical. It is currently unclear as to the mechanism of this enhancement, but it is likely linked to the immunogenicity of the envelopes themselves.

The goal of the ConB VLP and PolyB VLP vaccines was to induce a broadly reactive immune response. To measure the breadth of immunity, a panel of clade B isolates was selected that represent a general sample of all clade B isolates. Of particular interest were the isolates that were identified soon after infection, since these isolates are more representative of sexually transmitted viruses, which cause the majority of infections worldwide [183, 279, 281]. Statistically significant differences were not found between the ConB VLP and PolyB VLP vaccines for any given single isolate (Fig. 19). There was a trend towards higher responses with the PolyB VLP vaccine particularly against the early isolates. The PolyB VLP vaccine did induce a statistically significant increase in the breadth of envelope reactivity as measured by the Tukey's multiple comparison test. This statistical test takes into account the number of envelopes recognized as well as the strength of that recognition[91]. It is likely that this

increased breadth is due to the presentation of a more diverse set of epitopes as compared to the consensus vaccine. A consensus vaccine will present epitopes that are common to a large number of isolates, but it is possible that a polyvalent vaccine will present these common epitopes while also presenting a number of unique epitopes. These unique epitopes would then extend the breadth of Env recognition to include isolates that do not contain the most common epitopes, but do contain the unique epitopes.

The true test of any vaccine is its ability to protect against infection. To determine the protective effect of ConB VLP and PolyB VLP vaccines, all groups were challenged with multiple vaginal exposures to SHIV_{SF162p4}. This virus is heterologous to the ConB envelope and each envelope in the PolyB vaccine (Table 8). A vaginal challenge with SHIV_{SF162p4} with a total of four exposures resulted in a 75% infection rate in the Naïve group giving the baseline of comparison (Fig. 21). ConB VLP vaccination had no effect on infection outcome with 75% of the group becoming infected. Two of the infected NHPs had identical viral burdens as compared to the naïve group indicating a lack of protective effect. One NHP in the ConB VLP group did demonstrate a one log₁₀ reduction in viral load. This individual also demonstrated the highest anti-Gag antibody responses as well as the only anti-SF162 Env antibody responses in the ConB VLP group (Fig. 18 and 20). It is likely that these immune responses are the reason for the lower viral burden. The PolyB VLP vaccine demonstrated a clear protective effect by reducing the infection rate to 25% (Fig. 21). The one NHP who did become infected demonstrated a 2-log₁₀ reduction in viral burden over the course of infection indicating that the PolyB VLP vaccine was able to reduce the infection if not completely blocked. It is likely that the high levels of anti-Gag and anti-SF162 Env antibody levels induced by the PolyB VLP vaccine were able to induce this protection (Fig. 18 and 20). It is possible that the incomplete protection demonstrated by AH69

is due to its lack of anti-Env responses. AH69 had no detectable anti-SF162 antibody responses (Fig. 20) and was the only member of the PolyB VLP group to not recognize any of the envelopes tested (Fig. 19) thus limiting its immune protection to anti-Gag antibodies only. Previous studies have demonstrated that non-neutralizing antibodies are capable of inhibiting HIV-1 infection and replication in macrophages and immature dendritic cells[75]. These cells are the first cells exposed to the virus during mucosal transmission and play key roles in the early establishment of infection [285-286]. By blocking infection and transmission to these cells the PolyB vaccine is able to either completely block infection or significantly reduce the inoculating dose.

Upon the establishment of infection, all groups had similar immune responses. The rate and strength of these immune responses was identical across all groups and was only observed in those NHPs that had measurable vRNA levels (Fig. 24, 25 and 26). This indicates that these immune responses were generated in response to the infection alone and were not primed or affected by the vaccination state of the NHP. All post-challenge immune responses also correlated with viral burden throughout the disease course further indicating that they were dependent on the infection alone (Fig. 24). These results indicate that vaccination with either the ConB VLP or PolyB VLP vaccine did not affect post-challenge immune responses.

The use of a polyvalent vaccine demonstrated an increased ability to protect against a heterologous viral challenge as compared to a consensus vaccine. This is likely due to the increased levels of anti-Gag and anti-SF162 Env antibody responses that were generated. Future work is needed to induce stronger anti-Env immune responses pre-challenge. The virus-like particle platform is limited in the amount of Env on its surface. It is likely that priming the immune response by vaccinating with an Env only vaccine followed by boosting with a VLP will

facilitate the generation of a stronger anti-Env responses that may be enriched for neutralizing antibodies[274]. The current vaccine strategy also does not induce a cellular response (data not shown). Cellular immune responses may not block the initial infection, but they are capable of clearing already infected cells likely further decreasing the viral burden. To accomplish this either a larger dose of purified VLP or a viral-vector delivered VLP is needed[287]. The PolyB VLP vaccine used in this study demonstrates that a polyvalent vaccine is the best strategy to generate a broadly reactive immune response and that this immune response is capable of eliciting protection against a heterologous mucosal SHIV challenge.

6.0 SUMMARY AND DISCUSSION

There is a great need for the development of an effective HIV-1 vaccine. Each year, 3-4 million people are newly infected with HIV-1. Previous vaccine studies indicated that protection against infection is possible in the non-human primate model. None of these vaccines however have resulted in human protection. A large factor in this lack of protection is the impossibility to match the vaccine strain to the range of viral isolates that a person may be exposed. In most protective NHP trials, the vaccine strain and challenge strain have been exactly matched. Therefore, it is necessary to develop a vaccine that can induce the protective effect of a matched vaccine without being matched to the challenge virus.

The overall aim of this dissertation was to determine the best strategy for developing a broadly reactive immune response to HIV-1 envelope. Envelope was the primary target for this development as previous work has shown that antibodies directed towards envelope are capable of blocking infection if present at the site of infection in sufficient quantities. The first aim of this study was to determine the optimal form of envelope to use in a vaccination. The second aim was to compare the breadth of cellular immunity generated by consensus and polyvalent vaccines utilizing the delivery strategy found in aim 1. The third aim was to compare the breadth of humoral immunity generated by consensus and polyvalent vaccines and to determine their protective effect against a heterologous mucosal challenge. This work together has determined an optimal strategy for developing a broadly reactive HIV-1 vaccine.

The above work resulted in the development of a polyvalent clade B virus-like particle vaccine that is able to induce humoral immune responses that recognize a greater number of viral isolates as well as reduce the transmission rate of a heterologous challenge virus. The PolyB vaccine induced both SIV Gag and Env-binding antibody responses. While anti-VLP antibody responses that recognized cellular components of the VLP were generated, it is unlikely that these responses had any added effect on preventing infection beyond the immunity induced to Env. These components would have been identical between the ConB and PolyB VLP vaccines. However, the ConB VLP vaccine did not demonstrate any protective effect in the absence of anti-Env antibody responses. This indicates that the observed protection was most likely dependent on anti-Env antibody binding. It appears that these antibodies are binding to the virus upon viral exposure and blocking the virus's interaction with dendritic cells and monocytes thereby blocking the initial steps of infection. This reaction appears to be sufficient to completely block the infection as indicated by the lack of detectable viral RNA throughout infection and the fact that anti-viral immune responses are not boosted or generated after viral exposure. If the immune responses are not at a high enough titer however, this block can be overcome as demonstrated by AH69 within the PolyB group. AH69 had strong anti-Gag immune responses, but no detectable anti-Env responses. The lowered vRNA levels found within this NHP, indicate that the initial infection was only partially blocked. This partial block was able to reduce the infecting bolus of virus, but enough virus was able to gain access to induce a low level infection.

The most recent human HIV-1 vaccine trial is the first to indicate that a HIV-1 vaccine can induce a reduction in the rate of infection in humans[288]. This study utilized a vaccine containing three different Env isolates. A single Env, clade B, isolate as well as clade B Gag and

Pr were delivered via a canarypox vector followed by boosting with a two separate soluble gp120s, clade B and clade E. This vaccine resulted in a 31.2% reduction in the rate of infection. While this vaccine does contain multiple envelopes, which would facilitate increased antibody breadth, these envelopes were not specifically selected to generate broad coverage of the HIV-1 isolates found within Thailand thus potentially limiting the breadth generated. The use of more isolates would likely increase the protective effect observed by Thai vaccine by facilitating recognition of a greater number of viruses. This vaccine does indicate that immune responses directed to HIV-1 Env, Gag, and Pr are sufficient to induce protection in humans. These results support the further development of the PolyB VLP vaccine. The PolyB VLP vaccine induces immune responses to both HIV-1 Env and Gag. The use of the multiple envelopes also increases the breadth of the viral isolates recognized thus increasing the number of virus that would be protected against.

The PolyB VLP vaccine was able to induce immune responses which recognized a greater breadth of Env isolates and to induce protection against a heterologous viral challenge. One member of the PolyB VLP group was not protected. This NHP had the lowest immune responses observed for the group indicating that stronger induction of immune responses may generate greater levels of protection. In the mouse model, VLP vaccination induces strong cellular and humoral immune responses, but in the NHP model, only antibody responses to Gag and Env are generated. These responses remain strong for Gag, but anti-Env responses are only observed at moderate to low levels. It is likely that the differences seen between the two models are related to the dose of antigen delivered. In the mouse model, 40 µg of total VLP is delivered corresponding to approximately 1 µg of Env. In the NHP model, 250 µg of total VLP is delivered corresponding to approximately 6 µg of Env. The 200+ µg dose of Gag appears to be

sufficient for inducing strong antibody responses, but the 6 µg of Env appears to be below this level. The dosage of Env is capable of inducing low level antibody responses. To overcome this limited immune response, higher doses of VLP could be used. It would be beneficial to test increasing doses of VLP up to 1 mg per vaccination to determine if the limited anti-Env response could be improved. If a 1 mg dose is still low other methods would need to be explored as production limitations would greatly impede doses of greater than 1 mg.

One method of increasing the level of anti-Env antibodies generated would be to prime with a soluble Env followed by a VLP boost. Priming with a soluble Env would greatly increase the dose of Env delivered allowing for the establishment of high levels of anti-Env antibody responses. Previous studies have shown that strong antibody responses to Env can be generated in the NHP model through the use of purified protein, DNA vaccination or viral vectors. Boosting with the VLP will further enhance the antibody response. The boosting effect would be most dramatic for those antibodies which recognize Env on the surface of a viral particle. This is of particular importance because these antibodies are the ones that would be the most likely to bind Env on the surface of the virus upon infection.

Increases in the level and functionality of antibodies to Env and Gag would greatly enhance the protective effect of the polyvalent VLP vaccine. This enhancement would be due to increasing the antibody levels capable of blocking HIV-1 infection. If the initial infection is not blocked however it is likely that the vaccine will have no effect on the immune responses post-infection and they will not increase the rate of viral clearance similar to that seen in aim 3. The induction of cellular immune responses is needed to affect viral clearance upon infection. Vaccination with a viral vector has been shown to induce strong cellular immune responses. Modified Vaccinia Ankara (MVA) and canary pox vectors have been shown to induce strong

cellular immune responses to both Gag and Env. Both of these vectors would allow for the incorporation of the entire VLP expression cassette thus still facilitating correct viral processing of the VLP. The use of a polyvalent vaccine utilizing an Env prime followed by a viral vector boost encoding the VLP would induce both strong and broad humoral and cellular immunity generating the optimal conditions to block and clear a potential infection.

The vaccines studied in this dissertation were all given with CpG adjuvants. These adjuvants have been well established in mouse studies, but they have not been thoroughly studied in non-human primates. It is possible that another strategy of increasing the anti-Env antibody responses would be to use a different adjuvant. A small pilot study conducted in our laboratory indicates that using Inject Alum (Thermo Fisher Scientific, Rockford, IL) as an adjuvant with these VLP vaccines is able to induce stronger anti-Gag and anti-Env antibody responses. Therefore in future studies, Alum would be a better choice as adjuvant.

The use of a polyvalent vaccine is the best strategy to induce a broadly reactive immune response when measured within a single clade. It is not clear however if this strategy is capable of inducing cross-clade protection. Once an optimal vaccination regimen has been found, it would be beneficial to test a polyvalent vaccine against cross-clade challenge viruses to determine the range of protection. It is possible that a clade-based polyvalent vaccine will likely be limited in its cross-clade protection. This is due to the fact that the polyvalent vaccines are designed to address the diversity with the given target set. If designed for a specific clade, the diversity of that clade will be addressed, but the higher level of diversity found across clades would be unaccounted for. The design of a polyvalent vaccine to cover multiple clades would need to include isolates from all clades of interest. Computational modeling could be used to select isolates that in combination would give the greatest range of coverage. It is possible that

the number of isolates needed to generate sufficient coverage would be too great for use in a single vaccine. Multiple clade specific vaccines could be generated and given according to the specific clades prevalent in a given geographic area.

One of the greatest challenges with any vaccine is production and distribution. The release of the Human Papillomavirus (HPV) virus-like particle vaccines indicates that a virus-like particle-based vaccine can be produced in sufficient quantities and purified to a high standard for world-wide distribution. The two HPV vaccines, Gardasil[®] (Merck & Co. Inc. Whitehouse Station, NJ, USA) and Cervarix[™] (GlaxoSmithKline Biologicals, Rixensart, Belgium), are produced using two separate expression systems. Cervarix[™] utilizes a baculovirus expression system which requires multiple chromatographic and filtration techniques [289]. Gardasil[®] is produced using a yeast expression system in which the VLP is expressed by a recombinant *Sccharomyces pombe* vector [290]. Both of these systems produce high quantities of VLPs that are purified through multiple step processes before they are pure enough for human use. HIV-1 VLPs could be produced and purified using similar techniques. These vaccines are and would be highly expensive. However these costs are much lower than potential anti-retroviral drug treatments. It is likely that for an HIV-1 VLP vaccine to be distributed to economically depressed areas where it is most needed, governmental or charitable support would be needed.

The development of an HIV-1/AIDS vaccine requires that the vast viral diversity of HIV-1 isolates be addressed. The goal of this work was to determine an optimal strategy for developing such a vaccine. The results indicate that a polyvalent vaccine is better capable of inducing a broadly reactive immune response than a consensus vaccine. This increased breadth is linked to increases in the level of protection against heterologous viral challenges. Further

development of the polyvalent vaccine addressing the issues raised above will likely improve the immunogenicity and protective effect of this vaccine

This work is the starting point for developing a broadly reactive HIV/AIDS vaccine. These results indicate that the best strategy to carry forward is a polyvalent vaccine. While the best strategy was identified an optimal vaccine delivery regimen was not. Future work should be focused on optimizing the current PolyB Env VLP vaccine. All future studies will need to be completed in NHPs for the optimization of this vaccine. The findings within Aim1 and Aim2 highlight the differences in the immune responses generated in rodent systems as compared to non-human primates. As this vaccine is eventually intended for human use, its optimization should be completed in the non-human primate model as this better mimics the human immune response. First increasing the doses of antigen delivered either through the use of viral vectors or increased purification should be researched. Next increased immune responses to envelope should be investigated using gp140 trimers to prime the anti-Env immune response. Lastly other adjuvants should be used to facilitate the induction of stronger immune responses. After optimal anti-Env immune responses are generated, additional challenge studies should be initiated using SHIV_{SF162p4} as well as more pathogenic challenge viruses. Additionally cross-clade challenges should be completed to determine the level of cross-clade protection. It will be important to determine if multiple clade specific or multi-clade vaccines will be needed to protect against different clades.

This dissertation has shown that a Polyvalent clade B Env VLP-based vaccine is best able to generate a broadly reactive immune responses. These immune responses are able to reduce the rate of infection against a heterologous viral challenge. This work is the beginning in the development of a broadly reactive HIV-1 vaccine. Further work is needed to optimize the

immune responses generated by this vaccine to better strengthen the immune responses generated. It is likely based on the data presented above that strengthening of the immune responses will lead to a greater degree of protection.

7.0 APPENDIX

Shown below is the data from a pilot NHP study which was conducted at the University of Pittsburgh to ensure that the VLPs were immunogenic. All NHPs received four vaccination on four week intervals. All vaccines were given at a dose of 250 µg of VLP with 125 µg CpG adjuvant in 500 µl sterile saline delivered via the intramuscular route.

Table 10 Pitt Virus-like Particle Vaccines

Vaccine	Isolate Name	Geographic Location of Patient	Mode of Transmission ^a	Length of Infection ^b
Consensus B (Con B)	---	---	---	---
Polyvalent B (Poly B)	PVO.4	Italy	M-M	4 weeks
	RHPA4259.7	USA	M-F	< 8 weeks
	SC422661.8	Trinidad	F-M	4 weeks
	AC10.0.29	USA	M-M	4 weeks
HIV Gag p55 (Gag)	---	---	---	---

^a direction of sexual transmission: M-M male to male, M-F male to female, F-M female to male

^b length of infection is defined as time between last negative HIV-1 test and first positive HIV-1 test.

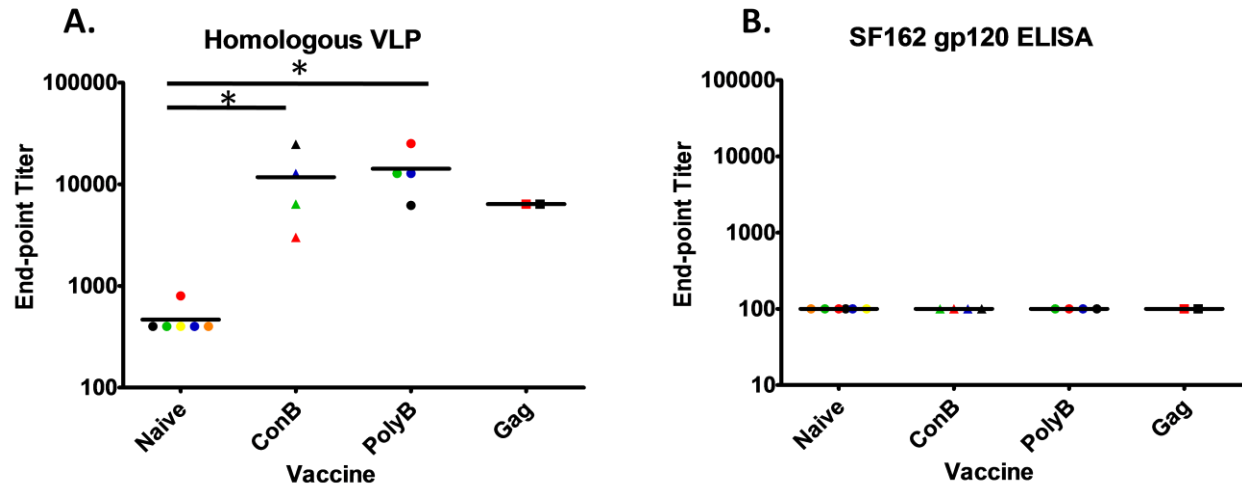


Figure 27 Pre-challenge antibody responses.

Serum samples were collected 2 weeks following the final vaccination. Serial dilutions were tested against either 50 ng of homologous VLP(A) or 50 ng of SF162 gp120(B). Results are shown as individual responses per group along with the average response. Statistics were completed using student's t-test. *p-value<.05

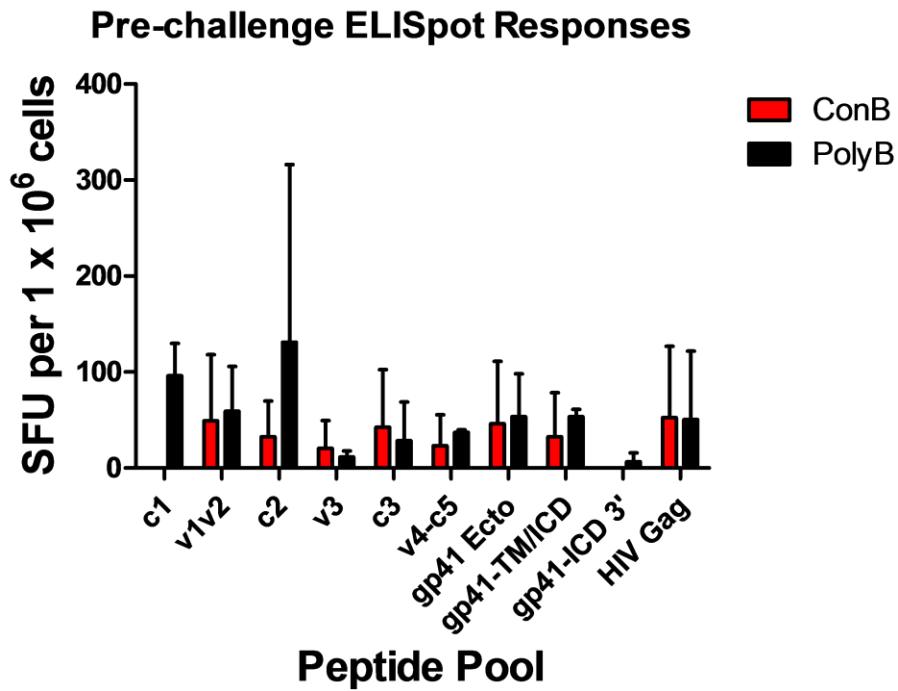


Figure 28 Pre-challenge Cellular Immune Responses.

Cellular immune responses were measured for PBMCs isolated two weeks following the final vaccination. Cells were tested against various peptide pools representing the different regions of Env (SF162 Env peptide set, NIH AIDS RRRP) as well as HIV Gag (NIH AIDS RRRP). Results are shown as the average responses per group (N=4).

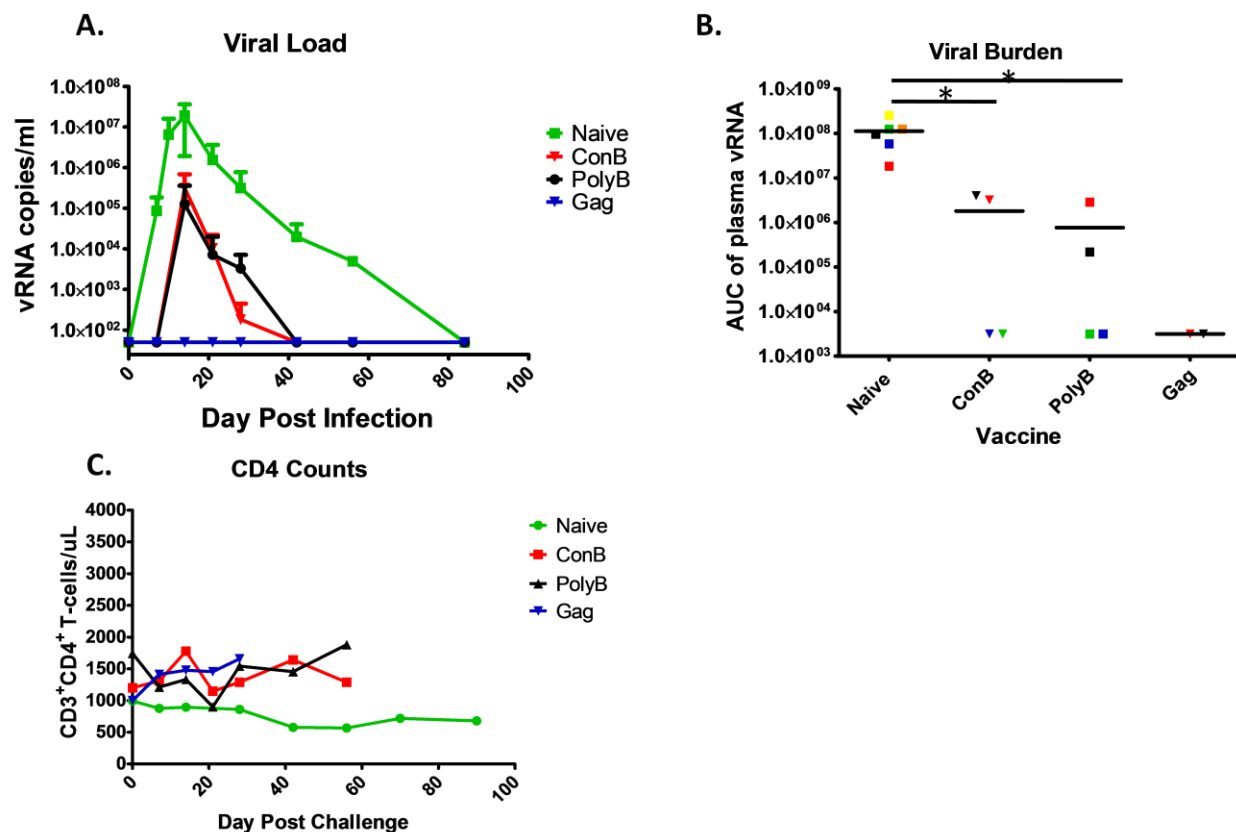


Figure 29 Pitt IR SHIV-SSF162p4 Challenge.

All groups were challenged intrarectally with 640 TCID₅₀ of SHIV_{SSF162p4} virus eight weeks following the final vaccination. (A) Viral loads are shown as the average \pm the SD for each group (Naive N=6, ConB N=4, PolyB N=4, Gag N=2). (B) The area under-the-curve for each individual viral load was taken to determine the viral burden over the course of infection. (C) CD4⁺ lymphocyte counts were observed throughout the infection course.

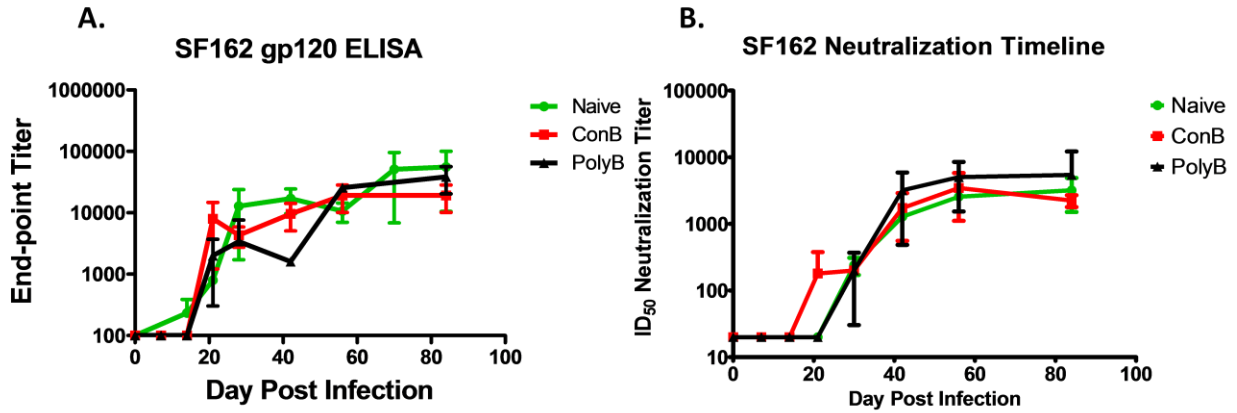


Figure 30 Post-Challenge Anti-Env antibody responses.

Antibody responses to SF162 Env were tested by ELISA (A) and Neutralization assay (B) throughout the post-challenge observation period. Results are shown as the average response from each infected NHP by group. Non-infected NHPs were not included as no changes in their immune responses were observed post-challenge.

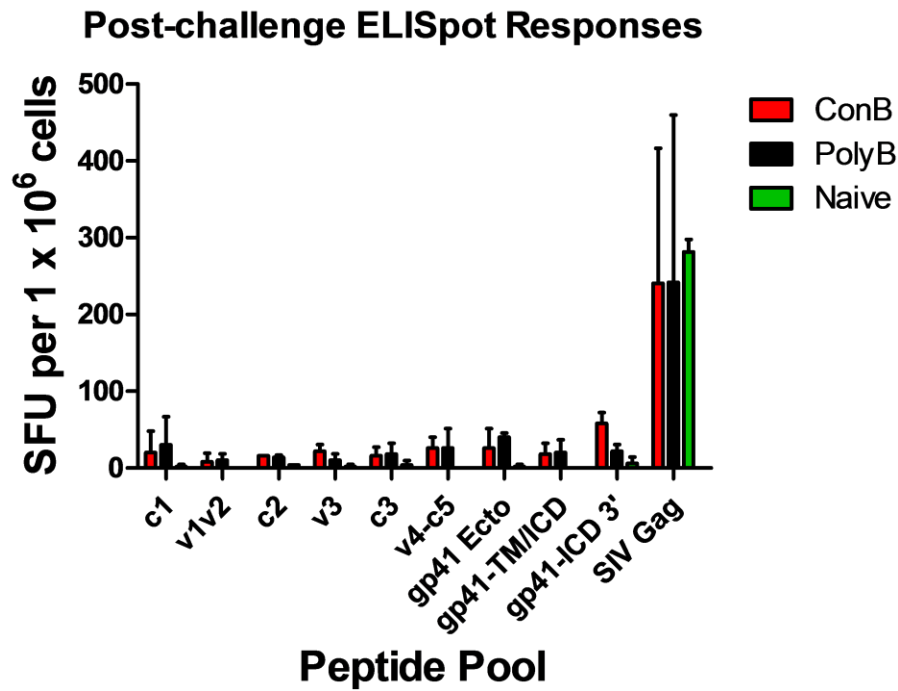


Figure 31 Post-challenge Cellular Responses.

Cellular immune responses were measured for PBMCs isolated at day 84 post-infection. Cells were tested against various peptide pools representing the different regions of Env (SF162 Env peptide set, NIH AIDS RRRP) as well as SIV Gag (NIH AIDS RRRP). Results are shown as the average response from each infected NHP by group.

From: Leela Ripton [l.ripton@futuremedicine.com]
To: McBurney, Sean P
Cc:
Subject: RE: Usage permission

Sent: Wed 9/30/2009 5:56 AM

Dear Sean,

Expert Reviews Ltd (Previously Future Drugs Ltd - Please see <http://www.future-science-group.com/news/1/78>) grants permission to reproduce the material as requested below with the following condition:

- 1) A citation of the article must be included with the reproduced material (see sample below).

Sample Citations:

Reproduced from Expert Rev. Vaccines 7(9), 1405-1417 (2008) with permission of Expert Reviews Ltd
Adapted from Expert Rev. Vaccines 7(9), 1405-1417 (2008) with permission of Expert Reviews Ltd

If you have any queries, please do not hesitate to contact me.

Kind regards,
Leela

Leela Ripton
Publishing Administrator

Future Science Group
Unitec House
2 Albert Place
Finchley
London
N3 1QB

**ELSEVIER LICENSE
TERMS AND CONDITIONS**

Feb 08, 2010

This is a License Agreement between Sean P McBurney ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard, Langford Lane Kidlington, Oxford, OX5 1GB, UK
Registered Company Number	1982084
Customer name	Sean P. McBurney
Customer address	3501 5th ave Pittsburgh, PA 15261
License Number	2278350848551
License date	Sep 29, 2009
Licensed content publisher	Elsevier
Licensed content publication	Virology
Licensed content title	Membrane embedded HIV-1 envelope on the surface of a virus-like particle elicits broader immune responses than soluble envelopes
Licensed content author	Sean P. McBurney, Kelly R. Young and Ted M. Ross
Licensed content date	20 February 2007
Volume number	358
Issue number	2
Pages	13
Type of Use	Thesis / Dissertation
Portion	Full article

**ELSEVIER LICENSE
TERMS AND CONDITIONS**

Feb 08, 2010

This is a License Agreement between Sean P McBurney ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard, Langford Lane Kidlington, Oxford, OX5 1GB, UK
Registered Company Number	1982084
Customer name	Sean P McBurney
Customer address	3501 5th ave Pittsburgh, PA 15261
License Number	2278350561273
License date	Sep 29, 2009
Licensed content publisher	Elsevier
Licensed content publication	Vaccine
Licensed content title	Human immunodeficiency virus-like particles with consensus envelopes elicited broader cell-mediated peripheral and mucosal immune responses than polyvalent and monovalent Env vaccines
Licensed content author	Sean P. McBurney and Ted M. Ross
Licensed content date	9 July 2009
Volume number	27
Issue number	32
Pages	13
Type of Use	Thesis / Dissertation
Portion	Full article

- [1] UNAIDS. AIDS epidemic update: November 2007: Joint United Nations Programme on HIV/AIDS and World Health Organization; 2007 November 2007.
- [2] Letvin NL. Progress and obstacles in the development of an AIDS vaccine. *Nature Reviews Immunology* 2006 Dec;6(12):930-9.
- [3] Sekaly R-P. The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development? *J Exp Med* 2008 January 21, 2008;205(1):7-12.
- [4] McMichael AJ. HIV VACCINES. *Annual Review of Immunology* 2006;24(1):227-55.
- [5] Thorner AR, Barouch DH. HIV-1 Vaccine Development: Progress and Prospects. *Current infectious disease reports* 2007(9):71-5.
- [6] Freed EO, Martin MA. *Fields Virology*. 4 ed. Philadelphia: Lippincott Williams and Wilkins, 2001.
- [7] Hallenberger S, Bosch V, Angliker H, Shaw E, Klenk H-D, Garten W. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. *Nature* 1992;360(6402):358-61.
- [8] Prabakaran P, Dimitrov AS, Fouts TR, Dimitrov DS, KuanTeh J. Structure and Function of the HIV Envelope Glycoprotein as Entry Mediator, Vaccine Immunogen, and Target for Inhibitors. *Advances in Pharmacology: Academic Press*, 2007: 33-97.
- [9] Gaschen B, Taylor J, Yusim K, Foley B, Gao F, Lang D, et al. Diversity Considerations in HIV-1 Vaccine Selection. *Science* 2002 June 28, 2002;296(5577):2354-60.
- [10] Robertson DL, J.P. Anderson, J.A. Bradac, J.K. Carr, B. Foley, R.K. Funkhouser, F. Gao, B.H. Hahn, M.L. Kalish, C. Kuiken, G.H. Learn, T. Leitner, F. McCutchan, S. Osmanov, M. Peeters, D. Pieniazek, M. Salminen, P.M. Sharp, S. Wolinsky, and B. Korber. *A Reference Guide to HIV-1 Classification*. 1999.
- [11] Johnston MI. Progress in HIV vaccine development. *Curr Opin Pharmacol* 2001;1:504-10.
- [12] Berman P, Gregory T, Riddle L, Nakamura G, Champe M, Porter J, et al. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature* 1990;345:622-5.
- [13] Polacino P. Limited breadth of protective immunity elicited by SIVmne gp160 vaccines in combination immunization regimen. *J Virol* 1999;73:618-30.
- [14] Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN, Gregory TJ. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *Journal of Biological Chemistry* 1990 June 25, 1990;265(18):10373-82.
- [15] Allan JS, Coligan JE, Barin F, McLane MF, Sodroski JG, Rosen CA, et al. Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. *Science* 1985 May 31;228(4703):1091-4.
- [16] Earl PL, Moss B, Doms RW. Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. *J Virol* 1991;65(4):2047-55.
- [17] Robey WG, Safai B, Oroszlan S, Arthur LO, Gonda MA, Gallo RC, et al. Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients. *Science* 1985 May 3;228(4699):593-5.
- [18] Veronese FD, DeVico AL, Copeland TD, Oroszlan S, Gallo RC, Sarngadharan MG. Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV envelope gene. *Science* 1985 Sep 27;229(4720):1402-5.

- [19] Chan DC, Fass D, Berger JM, Kim PS. Core structure of gp41 from the HIV envelope glycoprotein. *Cell* 1997 Apr 18;89(2):263-73.
- [20] Tan K, Liu J, Wang J, Shen S, Lu M. Atomic structure of a thermostable subdomain of HIV-1 gp41. *Proc Natl Acad Sci U S A* 1997 Nov 11;94(23):12303-8.
- [21] Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC. Atomic structure of the ectodomain from HIV-1 gp41. *Nature* 1997 May 22;387(6631):426-30.
- [22] Yang X, Farzan M, Wyatt R, Sodroski J. Characterization of stable, soluble trimers containing complete ectodomains of human immunodeficiency virus type 1 envelope glycoproteins. *J Virol* 2000;74(12):5716-25.
- [23] Chao SF, Chan VL, Juranka P, Kaplan AH, Swanstrom R, Hutchison CA, 3rd. Mutational sensitivity patterns define critical residues in the palm subdomain of the reverse transcriptase of human immunodeficiency virus type 1. *Nucleic Acids Res* 1995 Mar 11;23(5):803-10.
- [24] Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, et al. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 1996;381(6584):661-6.
- [25] Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, Peiper SC, et al. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* 1996 Jun 28;85(7):1149-58.
- [26] Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, et al. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 1996 Jun 20;381(6584):667-73.
- [27] Freed EO. Characterization of the fusion domain of the HIV-1 envelope glycoprotein gp41. *Proc Natl Acad Sci U S A* 1990;87:4650-4.
- [28] Helseth E, Olshevsky U, Gabuzda D, Ardman B, Haseltine W, Sodroski J. Changes in the transmembrane region of the human immunodeficiency virus type 1 gp41 envelope glycoprotein affect membrane fusion. *J Virol* 1990 Dec;64(12):6314-8.
- [29] Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR. Change in Coreceptor Use Correlates with Disease Progression in HIV-1-Infected Individuals. *J Exp Med* 1997 February 17, 1997;185(4):621-8.
- [30] Curran JW, Morgan WM, Hardy AM, Jaffe HW, Darrow WW, Dowdle WR. The epidemiology of AIDS: current status and future prospects. *Science* 1985 Sep 27;229(4720):1352-7.
- [31] Mellors JW, Kingsley LA, Rinaldo CR, Jr., Todd JA, Hoo BS, Kokka RP, et al. Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. *Ann Intern Med* 1995 Apr 15;122(8):573-9.
- [32] Mellors JW, Rinaldo CR, Jr., Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science* 1996 May 24;272(5265):1167-70.
- [33] Schacker TW, Hughes JP, Shea T, Coombs RW, Corey L. Biological and virologic characteristics of primary HIV infection. *Ann Intern Med* 1998 Apr 15;128(8):613-20.
- [34] Niu MT, Stein DS, Schnittman SM. Primary human immunodeficiency virus type 1 infection: review of pathogenesis and early treatment intervention in humans and animal retrovirus infections. *J Infect Dis* 1993;168(6):1490-501.
- [35] McSherry GD. Human immunodeficiency-virus-related pulmonary infections in children. *Semin Respir Infect* 1996;11(3):173-83.

- [36] Dillingham RA, Lima AA, Guerrant RL. Cryptosporidiosis: epidemiology and impact. *Microbes Infect* 2002;4(10):1059-66.
- [37] Samuel R, Bettiker RL, Suh B. AIDS related opportunistic infections, going but not gone. *Arch Pharm Res* 2002;25(3):215-28.
- [38] Collins KR, Quinones-Mateu ME, Toossi Z, Arts EJ. Impact of tuberculosis on HIV-1 replication, diversity, and disease progression. *AIDS Rev* 2002;4(3):165-76.
- [39] Furrer H, Fux C. Opportunistic infections: an update. *J HIV Ther* 2002;7(1):2-7.
- [40] Otieno MW, Banura C, Katongole-Mbidde E, Johnson JL, Ghannoum M, Dowlati A, et al. Therapeutic challenges of AIDS-related non-Hodgkin's lymphoma in the United States and East Africa. *J Natl Cancer Inst* 2002;94(10):718-32.
- [41] Loy CT, Brew BJ. Treatment of opportunistic infections and lymphoma in the nervous system. *J HIV Ther* 2002;7(1):21-5.
- [42] Hermans P. Kaposi's sarcoma in HIV-infected patients: treatment options. *HIV Med* 2000;1(3):137-42.
- [43] Boshoff C, Weiss R. AIDS-related malignancies. *Nat Rev Cancer* 2002;2(5):373-82.
- [44] Barry AP, Silvestri G, Safrit JT, Sumpter B, Kozyr N, McClure HM, et al. Depletion of CD8+ Cells in Sooty Mangabey Monkeys Naturally Infected with Simian Immunodeficiency Virus Reveals Limited Role for Immune Control of Virus Replication in a Natural Host Species. *J Immunol* 2007 June 15, 2007;178(12):8002-12.
- [45] Murthy KK, Cobb EK, Rouse SR, McClure HM, Payne JS, Salas MT, et al. Active and passive immunization against HIV type 1 infection in chimpanzees. *AIDS Research & Human Retroviruses* 1998;14 Suppl 3:S271-6.
- [46] Conley AJ, Kessler JA, II, Boots LJ, McKenna PM, Schleif WA, Emini EA, et al. The consequence of passive administration of an anti-human immunodeficiency virus type 1 neutralizing monoclonal antibody before challenge of chimpanzees with a primary virus isolate. *Journal of Virology* 1996;70(10):6751-8.
- [47] Clements JE, Montelaro RC, Zink MC, Amedee AM, Miller S, Trichel AM, et al. Cross-protective immune responses induced in rhesus macaques by immunization with attenuated macrophage-tropic simian immunodeficiency virus. *Journal of Virology* 1995;69(5):2737-44.
- [48] Gardner M, Rosenthal A, Jennings M, Yee J, Antipa L, Robinson E, Jr. Passive immunization of rhesus macaques against SIV infection and disease. *AIDS Research & Human Retroviruses* 1995;11(7):843-54.
- [49] Haigwood NL, Watson A, Sutton WF, McClure J, Lewis A, Ranchalis J, et al. Passive immune globulin therapy in the SIV/macaque model: early intervention can alter disease profile. *Immunology Letters* 1996;51(1-2):107-14.
- [50] Van Rompay KK, Berardi CJ, Dillard-Telm S, Tarara RP, Canfield DR, Valverde CR, et al. Passive immunization of newborn rhesus macaques prevents oral simian immunodeficiency virus infection. *Journal of Infectious Diseases* 1998;177(5):1247-59.
- [51] Mascola JR, Lewis MG, Stiegler G, Harris D, VanCott TC, Hayes D, et al. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *Journal of Virology* 1999;73(5):4009-18.
- [52] Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, et al. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nature Medicine* 2000;6(2):207-10.

- [53] Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu W, Ayehunie S, et al. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nature Medicine* 2000;6(2):200-6.
- [54] Foresman L, Jia F, Li Z, Wang C, Stephens EB, Sahni M, et al. Neutralizing antibodies administered before, but not after, virulent SHIV prevent infection in macaques. *AIDS Research & Human Retroviruses* 1998;14(12):1035-43.
- [55] Li A, Baba TW, Sodroski J, Zolla-Pazner S, Gorny MK, Robinson J, et al. Synergistic neutralization of a chimeric SIV/HIV type 1 virus with combinations of human anti-HIV type 1 envelope monoclonal antibodies or hyperimmune globulins. *AIDS Research & Human Retroviruses* 1997;13(8):647-56.
- [56] Li A, Katinger H, Posner MR, Cavacini L, Zolla-Pazner S, Gorny MK, et al. Synergistic neutralization of simian-human immunodeficiency virus SHIV-vpu+ by triple and quadruple combinations of human monoclonal antibodies and high-titer anti-human immunodeficiency virus type 1 immunoglobulins. *Journal of Virology* 1998;72(4):3235-40.
- [57] Shibata R, Igarashi T, Haigwood N, Buckler-White A, Ogert R, Ross W, et al. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys.[comment]. *Nature Medicine* 1999;5(2):204-10.
- [58] Barnett SW. Vaccination with HIV-1 gp120 DNA induces immune responses that are boosted by a recombinant gp120 protein subunit. *Vaccine* 1997;15:869-73.
- [59] Burton DRM, D.C. The antibody response in HIV-1 infection. *AIDS* 1997;11:S87-S98.
- [60] Graham BS. Determinants of antibody response after recombinant gp160 boosting in vaccinia-naïve volunteers premed with gp160-recombinant vaccinia virus. *J Infect Dis* 1994;170:782-6.
- [61] Mascola JR, Snyder SW, Weislow OS, Belay SM, Belshe RB, Schwartz DH. Immunization with envelope subunit vaccine products elicits neutralizing antibodies against laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. *J Infect Dis* 1996;173:340-8.
- [62] Mascola JR. Protection of macaques against vaginal transmission of pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med* 2000;6:207-10.
- [63] Parren PW. Neutralization of HIV-1 by antibody to gp120 is determined primarily by occupancy of sites on the virion irrespective of epitope specificity. *J Virol* 1998;73:3512-9.
- [64] Van Cott TC. Antibodies with specificity to native gp120 and neutralization activity against primary HIV-1 isolates elicited by immunization with oligomeric gp160. *J Virol* 1997;71:4319-30.
- [65] Yang X. Characterization of stable, soluble trimer containing complete ectodomains of HIV-1 envelope glycoprotein. *J Virol* 2000;74:5716-25.
- [66] Cho MW. Polyvalent envelope glycoprotein vaccine elicits a broader neutralizing antibody response but is unable to provide sterilizing protection against heterologous Simian/human immunodeficiency virus infection in pigtailed macaques. *J Virol* 2001;75:2224-34.
- [67] Cole KS, Rowles JL, Murphey-Corb M, Clements JE, Robinson J, Montelaro RC. A model for the maturation of protective antibody responses to SIV envelope proteins in experimentally immunized monkeys. *Journal of Medical Primatology* 1997;26(1-2):51-8.
- [68] Cole KS, Murphey-Corb M, Narayan O, Joag SV, Shaw GM, Montelaro RC. Common themes of antibody maturation to simian immunodeficiency virus, simian-human

immunodeficiency virus, and human immunodeficiency virus type 1 infections. *Journal of Virology* 1998;72(10):7852-9.

[69] Fuller DH, Simpson L, Cole KS, Clements JE, Panicali DL, Montelaro RC, et al. Gene gun-based nucleic acid immunization alone or in combination with recombinant vaccinia vectors suppresses virus burden in rhesus macaques challenged with a heterologous SIV. *Immunology & Cell Biology* 1997;75(4):389-96.

[70] Johnson RP, Lifson JD, Czajak SC, Cole KS, Manson KH, Glickman R, et al. Highly attenuated vaccine strains of simian immunodeficiency virus protect against vaginal challenge: inverse relationship of degree of protection with level of attenuation. *Journal of Virology* 1999;73(6):4952-61.

[71] Hammond SA, Cook SJ, Lichtenstein DL, Issel CJ, Montelaro RC. Maturation of the cellular and humoral immune responses to persistent infection in horses by equine infectious anemia virus is a complex and lengthy process. *Journal of Virology* 1997;71(5):3840-52.

[72] Montelaro RC, Cole KS, Hammond SA. Maturation of immune responses to lentivirus infection: implications for AIDS vaccine development. *AIDS Research & Human Retroviruses* 1998;14 Suppl 3:S255-9.

[73] Van Rompay K, Greenier, JL, Cole, KS, Earl, P, Moss, B, Steckbeck, JD, Pahar, B, Rourke, T, Montelaro, RC, Canfield, DR, Tarara, RP, Miller, C, McChesney, MB, Marthas, ML. Immunization of newborn rhesus macaques with simian immunodeficiency virus (SIV) vaccines prolongs survival after oral challenge with virulent SIVmac251. *J Virol* 2003;77:179-90.

[74] Emini EA, Schleif WA, Nunberg JH, Conley AJ, Eda Y, Tokiyoshi S, et al. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. *Nature* 1992;355(6362):728.

[75] Holl V, Peressin M, Decoville T, Schmidt S, Zolla-Pazner S, Aubertin A-M, et al. Nonneutralizing Antibodies Are Able To Inhibit Human Immunodeficiency Virus Type 1 Replication in Macrophages and Immature Dendritic Cells. *J Virol* 2006 June 15, 2006;80(12):6177-81.

[76] Mascola JR, Lewis MG, Stiegler G, Harris D, VanCott TC, Hayes D, et al. Protection of Macaques against Pathogenic Simian/Human Immunodeficiency Virus 89.6PD by Passive Transfer of Neutralizing Antibodies. *J Virol* 1999 May 1, 1999;73(5):4009-18.

[77] Manrique M, Micewicz E, Kozlowski PA, Wang S-W, Aurora D, Wilson RL, et al. DNA-MVA Vaccine Protection after X4 SHIV Challenge in Macaques Correlates with Day-of-Challenge Antiviral CD4+ Cell-Mediated Immunity Levels and Postchallenge Preservation of CD4+ T Cell Memory. *AIDS Research and Human Retroviruses* 2008;24(3):505-19.

[78] Nilsson C, Makitalo B, Thorstensson R, Norley S, Binninger-Schinzel D, Cranage M, et al. Live attenuated simian immunodeficiency virus (SIV)mac in macaques can induce protection against mucosal infection with SIVsm. [Article]: *AIDS* November 1998;12(17):2261-2270.

[79] Wyand MS, Manson K, Montefiori DC, Lifson JD, Johnson RP, Desrosiers RC. Protection by Live, Attenuated Simian Immunodeficiency Virus against Heterologous Challenge. *J Virol* 1999 October 1, 1999;73(10):8356-63.

[80] Wyand MS, Manson KH, Garcia-Moll M, Montefiori D, Desrosiers RC. Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. *J Virol* 1996 June 1, 1996;70(6):3724-33.

[81] Xu R, Srivastava IK, Kuller L, Zarkikh I, Kraft Z, Fagrouch Z, et al. Immunization with HIV-1 SF162-derived Envelope gp140 proteins does not protect macaques from heterologous simian-human immunodeficiency virus SHIV89.6P infection. *Virology* 2006;349(2):276-89.

- [82] Someya K, Cecilia D, Ami Y, Nakasone T, Matsuo K, Burda S, et al. Vaccination of Rhesus Macaques with Recombinant Mycobacterium bovis Bacillus Calmette-Guerin Env V3 Elicits Neutralizing Antibody-Mediated Protection against Simian-Human Immunodeficiency Virus with a Homologous but Not a Heterologous V3 Motif. *J Virol* 2005 February 1, 2005;79(3):1452-62.
- [83] Nickle DC, Jensen MA, Gottlieb GS, Shriner D, Learn GH, Rodrigo AG, et al. Consensus and Ancestral State HIV Vaccines. *Science* 2003 March 7, 2003;299(5612):1515c-8.
- [84] Weaver EA, Lu Z, Camacho ZT, Moukdar F, Liao H-X, Ma B-J, et al. Cross-Subtype T-Cell Immune Responses Induced by a Human Immunodeficiency Virus Type 1 Group M Consensus Env Immunogen. *J Virol* 2006 July 15, 2006;80(14):6745-56.
- [85] Gao F, Weaver EA, Lu Z, Li Y, Liao H-X, Ma B, et al. Antigenicity and Immunogenicity of a Synthetic Human Immunodeficiency Virus Type 1 Group M Consensus Envelope Glycoprotein. *J Virol* 2005 January 15, 2005;79(2):1154-63.
- [86] Korber BT, Brander, C., Haynes, B. HIV Molecular Immunology 2002. 2002;Theoretical Biology and Biophysics Group:Los Alamos National Laboratory.
- [87] Zhan X. Minor components of a multi-envelope HIV vaccine are recognized by type-specific T-helper cells. *Vaccine* 2004;22:1206-13.
- [88] Yusim K, Kesmir, C., Gaschen, B. Clustering patterns of cytotoxic T-lymphocyte epitopes in human immunodeficiency virus type 1 (HIV-1) proteins reveal imprints of immune evasion on HIV-1 global variation. *J Virol* 2002;76:8757-68.
- [89] Zolla-Pazner S. Identifying epitopes of HIV-1 that induce protective antibodies. *Nat Rev Immunol* 2004;4:199-210.
- [90] Doria-Rose NA, Learn GH, Rodrigo AG, Nickle DC, Li F, Mahalanabis M, et al. Human Immunodeficiency Virus Type 1 Subtype B Ancestral Envelope Protein Is Functional and Elicits Neutralizing Antibodies in Rabbits Similar to Those Elicited by a Circulating Subtype B Envelope. *J Virol* 2005 September 1, 2005;79(17):11214-24.
- [91] Kothe DL, Decker JM, Li Y, Weng Z, Bibollet-Ruche F, Zammit KP, et al. Antigenicity and immunogenicity of HIV-1 consensus subtype B envelope glycoproteins. *Virology* 2007;360(1):218-34.
- [92] Kothe DL, Li Y, Decker JM, Bibollet-Ruche F, Zammit KP, Salazar MG, et al. Ancestral and consensus envelope immunogens for HIV-1 subtype C. *Virology* 2006;352(2):438-49.
- [93] Scriba TJ, zur Megede J, Glashoff RH, Treurnicht FK, Barnett SW, van Rensburg EJ. Functionally-inactive and immunogenic Tat, Rev and Nef DNA vaccines derived from sub-Saharan subtype C human immunodeficiency virus type 1 consensus sequences. *Vaccine* 2005;23(9):1158.
- [94] Liao H-X, Sutherland LL, Xia S-M, Brock ME, Searce RM, Vanleeuwen S, et al. A group M consensus envelope glycoprotein induces antibodies that neutralize subsets of subtype B and C HIV-1 primary viruses. *Virology* 2006;353(2):268-82.
- [95] Davies MN, Flower DR. Harnessing bioinformatics to discover new vaccines. *Drug Discovery Today* 2007;12(9-10):389-95.
- [96] Burgers WA, van Harmelen JH, Shephard E, Adams C, Mgwebi T, Bourn W, et al. Design and preclinical evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial. *J Gen Virol* 2006 February 1, 2006;87(2):399-410.
- [97] De Groot AS, Bishop EA, Khan B, Lally M, Marcon L, Franco J, et al. Engineering immunogenic consensus T helper epitopes for a cross-clade HIV vaccine. *Methods* 2004;34(4):476.

- [98] Hamano T, Sawanpanyalert P, Yanai H, Piyaworawong S, Hara T, Sapsutthipas S, et al. Determination of HIV Type 1 CRF01_AE gag p17 and env-V3 Consensus Sequences for HIV/AIDS Vaccine Design. *AIDS Research and Human Retroviruses* 2004;20(3):337-40.
- [99] Hanke T, McMichael AJ, Mwau M, Wee EGT, Ceberej I, Patel S, et al. Development of a DNA-MVA/HIVA vaccine for Kenya. *Vaccine* 2002;20(15):1995.
- [100] Krohn K, Stanescu I, Blazevic V, Vesikari T, Ranki A, Ustav M. A DNA HIV-1 vaccine based on a fusion gene expressing non-structural and structural genes of consensus sequence of the A-C subtypes and the ancestor sequence of the F-H subtypes. Preclinical and clinical studies. *Microbes and Infection* 2005;7(14):1405.
- [101] Kumar S, Aggarwal P, Vajpayee M, Pandey RM, Seth P. Development of a candidate DNA/MVA HIV-1 subtype C vaccine for India. *Vaccine* 2006;24(14):2585.
- [102] Malm M, Rollman E, Ustav M, Hinkula J, Krohn K, Wahren B, et al. Cross-Clade Protection Induced by Human Immunodeficiency Virus-1 DNA Immunogens Expressing Consensus Sequences of Multiple Genes and Epitopes From Subtypes A, B, C, and FGH. *Viral Immunology* 2005;18(4):678-88.
- [103] Nkolola J, EG-T Wee, E-J Im, CP Jewell, N Chen, X-N Xu, AJ McMichael and T Hanke. Engineering RENTA, a DNA prime-MVA boost HIV vaccine tailored for Eastern and Central Africa. *Gene Therapy* 2004 July 2004;11(13):1069-80.
- [104] Thomson SA, Jaramillo AB, Shoobridge M, Dunstan KJ, Everett B, Ranasinghe C, et al. Development of a synthetic consensus sequence scrambled antigen HIV-1 vaccine designed for global use. *Vaccine* 2005;23(38):4647.
- [105] Slobod K, C Coleclough, M Bonsignori, SA Brown, X Zhan, S Surman, A Zirkel, BG Jones, RE Sealy, J Stambas, B Brown, TD Lockey, PJ Freiden, PC Doherty, JL Blanchard, LN Martin, JL Hurwitz. HIV Vaccine Rationale, Design and Testing. *Current HIV Research* 2005 April;3(2):107-12.
- [106] Azizi A, Anderson D, Ghorbani M, Gee K, Diaz-Mitoma F. Immunogenicity of a polyvalent HIV-1 candidate vaccine based on fourteen wild type gp120 proteins in golden hamsters. *BMC Immunology* 2006;7(1):25.
- [107] Chakrabarti BK, Ling X, Yang Z-Y, Montefiori DC, Panet A, Kong W-P, et al. Expanded breadth of virus neutralization after immunization with a multiclade envelope HIV vaccine candidate. *Vaccine* 2005;23(26):3434.
- [108] Ljungberg K, Rollman E, Eriksson L, Hinkula J, Wahren B. Enhanced Immune Responses After DNA Vaccination with Combined Envelope Genes from Different HIV-1 Subtypes. *Virology* 2002;302(1):44.
- [109] Rollman E JH, J Arteaga, B Zuber, A Kjerrstrom, M Liu, B Wahren, K Ljungberg. Multi-subtype gp160 DNA immunization induces broadly neutralizing anti-HIV antibodies. *Gene Therapy* 2004 July 2004;11(14):1146-54.
- [110] Wang S, Pal R, Mascola JR, Chou T-HW, Mboudjeka I, Shen S, et al. Polyvalent HIV-1 Env vaccine formulations delivered by the DNA priming plus protein boosting approach are effective in generating neutralizing antibodies against primary human immunodeficiency virus type 1 isolates from subtypes A, B, C, D and E. *Virology* 2006;350(1):34-47.
- [111] Brave A, Boberg A, Gudmundsdotter L, Rollman E, Hallermalm K, Ljungberg K, et al. A New Multi-clade DNA Prime/Recombinant MVA Boost Vaccine Induces Broad and High Levels of HIV-1-specific CD8⁺ T-cell and Humoral Responses in Mice. *Mol Ther* 2007;15(9):1724-33.

- [112] Brave A, Ljungberg K, Boberg A, Rollman E, Isaguliant M, Lundgren B, et al. Multigene/Multisubtype HIV-1 Vaccine Induces Potent Cellular and Humoral Immune Responses by Needle-Free Intradermal Delivery. *Molecular Therapy* 2005;12(6):1197.
- [113] Cristillo AD, Wang S, Caskey MS, Unangst T, Hocker L, He L, et al. Preclinical evaluation of cellular immune responses elicited by a polyvalent DNA prime/protein boost HIV-1 vaccine. *Virology* 2006;346(1):151.
- [114] Kong W-P, Huang Y, Yang Z-Y, Chakrabarti BK, Moodie Z, Nabel GJ. Immunogenicity of Multiple Gene and Clade Human Immunodeficiency Virus Type 1 DNA Vaccines. *J Virol* 2003 December 1, 2003;77(23):12764-72.
- [115] Pal R, Wang S, Kalyanaraman VS, Nair BC, Whitney S, Keen T, et al. Polyvalent DNA prime and envelope protein boost HIV-1 vaccine elicits humoral and cellular responses and controls plasma viremia in rhesus macaques following rectal challenge with an R5 SHIV isolate. *Journal of Medical Primatology* 2005;34(5-6):226-36.
- [116] Pal R, Wang S, Kalyanaraman VS, Nair BC, Whitney S, Keen T, et al. Immunization of rhesus macaques with a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 vaccine elicits protective antibody response against simian human immunodeficiency virus of R5 phenotype. *Virology* 2006 May 10;348(2):341-53.
- [117] Pal R, Yu Q, Wang S, Kalyanaraman VS, Nair BC, Hudacik L, et al. Definitive toxicology and biodistribution study of a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 (HIV-1) vaccine in rabbits. *Vaccine* 2006;24(8):1225.
- [118] Seaman MS, Xu L, Beaudry K, Martin KL, Beddall MH, Miura A, et al. Multiclade Human Immunodeficiency Virus Type 1 Envelope Immunogens Elicit Broad Cellular and Humoral Immunity in Rhesus Monkeys. *J Virol* 2005 March 1, 2005;79(5):2956-63.
- [119] Wang S, Kennedy JS, West K, Montefiori DC, Coley S, Lawrence J, et al. Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA prime-protein boost HIV-1 vaccine in healthy human volunteers. *Vaccine* 2008;26(8):1098-110.
- [120] Fischer W, Perkins S, Theiler J, Bhattacharya T, Yusim K, Funkhouser R, et al. Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants. *Nat Med* 2007;13(1):100-6.
- [121] Azizi A, Anderson DE, Torres JV, Ogrel A, Ghorbani M, Soare C, et al. Induction of Broad Cross-Subtype-Specific HIV-1 Immune Responses by a Novel Multivalent HIV-1 Peptide Vaccine in Cynomolgus Macaques. *J Immunol* 2008 February 15, 2008;180(4):2174-86.
- [122] Hanke T. STEP trial and HIV-1 vaccines inducing T-cell responses. *Expert Review of Vaccines* 2008;7(3):303-9.
- [123] Watkins DI, Burton DR, Kallas EG, Moore JP, Koff WC. Nonhuman primate models and the failure of the Merck HIV-1 vaccine in humans. *Nat Med* 2008;14(6):617-21.
- [124] Koff WC, Johnson PR, Watkins DI, Burton DR, Lifson JD, Hasenkrug KJ, et al. HIV vaccine design: insights from live attenuated SIV vaccines. *Nat Immunol* 2006;7(1):19-23.
- [125] Spearman P. HIV Vaccine Development: Lessons from the Past and Promise for the Future. *Current HIV Research* 2003;1(1):pp.101-20.
- [126] Hamer DH. Can HIV be cured? Mechanisms of HIV persistence and strategies to combat it. *Current HIV Research* 2004;2(2):99-111.
- [127] Conley AJ, Kessler Ja, II, Boots LJ, McKenna PM, Schleif WA, Emini EA, et al. The consequence of passive administration of an anti-human immunodeficiency virus type 1 neutralizing monoclonal antibody before challenge of chimpanzees with a primary virus isolate. *J Virol* 1996 October 1, 1996;70(10):6751-8.

- [128] Murthy KK, Cobb EK, Rouse SR, McClure HM, Payne JS, Salas MT, et al. Active and passive immunization against HIV type 1 infection in chimpanzees. *AIDS Res Hum Retroviruses* 1998;14(Suppl. 3):S271-S6.
- [129] Gardner M, Rosenthal, A, Jennings, M, Yee, J, Antipa, L, Robinson, E Jr. Passive immunization of rhesus macaques against SIV infection and disease. *AIDS Res Hum Retroviruses* 1995;11(7):843-54.
- [130] Haigwood NL, Watson A, Sutton WF, McClure J, Lewis A, Ranchalis J, et al. Passive immune globulin therapy in the SIV/macaque model: early intervention can alter disease profile. *Immunology Letters* 1996;51(1-2):107-14.
- [131] Foresman L, Jia, F, Li, Z, Wang, C, Stephens, EB, Sahni, M, Narayan, O, Joag, SV. Neutralizing antibodies administered before, but not after, virulent SHIV prevent infection in macaques. *AIDS Res Hum Retroviruses* 1998;14(12):1035-43.
- [132] Li A, Baba TW, Sodroski J, Zolla-Pazner S, Gorny MK, Robinson J, et al. Heterologous envelope immunogens contribute to AIDS vaccine protection in rhesus monkeys. *AIDS Res Hum Retroviruses* 1997;13(8):647-56.
- [133] Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, et al. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med* 2000;6(2):207-10.
- [134] Shibata R, Igarashi T, Haigwood N, Buckler-White A, Ogert R, Ross W, et al. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nat Med* 1999;5(2):204-10.
- [135] Young KR, James M. Smith and Ted M. Ross Characterization of a DNA vaccine expressing a human immunodeficiency virus-like particle. *Virology* 2004 1 October 2004;327(2):262-72.
- [136] Young KR, Ross TM. Elicitation of Immunity to HIV Type 1 Gag Is Determined by Gag Structure. *AIDS Research and Human Retroviruses* 2006;22(1):99-108.
- [137] Ellenberger D, Linda Wyatt, Bin Li, Suzan Buge, Nattawan Lanier, I. Vanessa Rodriguez, Carlos A. Sariol, Melween Martinez, Michael Monsour, Jennifer Vogt, James Smith, Ronald Otten, David Montefiori, Edmundo Kraiselburd, Bernard Moss, Harriet Robinson, Janet McNicholl and Salvatore Butera Comparative immunogenicity in rhesus monkeys of multi-protein HIV-1 (CRF02_AG) DNA/MVA vaccines expressing mature and immature VLPs. *Virology* 2005 15 September 2005;340(1):21-32.
- [138] Ellenberger D, Li B, Smith J, Yi H, Folks T, Robinson H, et al. Optimization of a multi-gene HIV-1 recombinant subtype CRF02_AG DNA vaccine for expression of multiple immunogenic forms. *Virology* 2004;319(1):118-30.
- [139] Hammonds J, Chen X, Ding L, Fouts T, De Vico A, zur Megede J, et al. Gp120 stability on HIV-1 virions and Gag-Env pseudovirions is enhanced by an uncleaved Gag core. *Virology* 2003;314(2):636-49.
- [140] Smith JM, Amara RR, Campbell D, Xu Y, Patel M, Sharma S, et al. DNA/MVA vaccine for HIV type 1: effects of codon-optimization and the expression of aggregates or virus-like particles on the immunogenicity of the DNA prime. *AIDS Res Hum Retroviruses* 2004;20(12):1335-47.
- [141] Amara RR, Francois Villinger, John D. Altman, Shari L. Lydya, Shawn P. O'Neil, Silvija I. Staprans, David C. Montefiori Yan Xu, James G. Herndon, Linda S. Wyatt, Maria Angelito Candido, Natalia L. Kozyr, Patricia L. Earl, James M. Smith, Hak-Ling Ma, Bennett D. Grimm, Michael L. Hulsey, Harold M. McClure, Janet M. McNicholl, Bernard Moss and Harriet L.

- Robinson. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Vaccine* 2002 6 May 2002;20(15):1949-55.
- [142] Wyatt LS, Earl PL, Liu JY, Smith JM, Montefiori DC, Robinson HL, et al. Multiprotein HIV type 1 clade B DNA and MVA vaccines: construction, expression, and immunogenicity in rodents of the MVA component. *AIDS Res Hum Retroviruses* 2004;20(6):645-53.
- [143] Egan MA. Current Prospects for the Development of a Therapeutic Vaccine for the Treatment of HIV Type 1 Infection. *AIDS Research and Human Retroviruses* 2004;20(8):794-806.
- [144] Haigwood N. Predictive value of primate models for AIDS. *AIDS Rev* 2004;6(4):187-98.
- [145] Sauter SL, Rahman A, Muralidhar G. Non-replicating viral vector-based AIDS vaccines: interplay between viral vectors and the immune system. *Current HIV Research* 2005;3(2):157-81.
- [146] Amara RR, Smith JM, Staprans SI, Montefiori DC, Villinger F, Altman JD, et al. Critical Role for Env as well as Gag-Pol in Control of a Simian-Human Immunodeficiency Virus 89.6P Challenge by a DNA Prime/Recombinant Modified Vaccinia Virus Ankara Vaccine. *J Virol* 2002 June 15, 2002;76(12):6138-46.
- [147] Letvin NL, Huang Y, Chakrabarti BK, Xu L, Seaman MS, Beaudry K, et al. Heterologous Envelope Immunogens Contribute to AIDS Vaccine Protection in Rhesus Monkeys. *J Virol* 2004 July 15, 2004;78(14):7490-7.
- [148] Forthal DN, Landucci G, Daar ES. Antibody from Patients with Acute Human Immunodeficiency Virus (HIV) Infection Inhibits Primary Strains of HIV Type 1 in the Presence of Natural-Killer Effector Cells. *J Virol* 2001 August 1, 2001;75(15):6953-61.
- [149] Forthal Donald N, Landucci G, Haubrich R, Keenan B, Kuppermann Baruch D, Tilles Jeremiah G, et al. Antibody Dependent Cellular Cytotoxicity Independently Predicts Survival in Severely Immunocompromised Human Immunodeficiency Virus Infected Patients. *The Journal of Infectious Diseases* 1999;180(4):1338-41.
- [150] Gomez-Roman VR, Patterson LJ, Venzon D, Liewehr D, Aldrich K, Florese R, et al. Vaccine-Elicited Antibodies Mediate Antibody-Dependent Cellular Cytotoxicity Correlated with Significantly Reduced Acute Viremia in Rhesus Macaques Challenged with SIVmac251. *J Immunol* 2005 February 15, 2005;174(4):2185-9.
- [151] Peng B, Wang LR, Gomez-Roman VR, Davis-Warren A, Montefiori DC, Kalyanaraman VS, et al. Replicating Rather than Nonreplicating Adenovirus-Human Immunodeficiency Virus Recombinant Vaccines Are Better at Eliciting Potent Cellular Immunity and Priming High-Titer Antibodies. *J Virol* 2005 August 15, 2005;79(16):10200-9.
- [152] Farzan M, Choe H, Desjardins E, Sun Y, Kuhn J, Cao J, et al. Stabilization of Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Trimers by Disulfide Bonds Introduced into the gp41 Glycoprotein Ectodomain. *J Virol* 1998 September 1, 1998;72(9):7620-5.
- [153] Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 1998;393(6686):648-59.
- [154] Wyatt R, Sodroski J. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* 1998;280(5371):1884-8.
- [155] Beddows S, Schulke N, Kirschner M, Barnes K, Franti M, Michael E, et al. Evaluating the Immunogenicity of a Disulfide-Stabilized, Cleaved, Trimeric Form of the Envelope Glycoprotein Complex of Human Immunodeficiency Virus Type 1. *J Virol* 2005 July 15, 2005;79(14):8812-27.

- [156] Bower JF, Sanders KL, Ross TM. C3d enhances immune responses using low doses of DNA expressing the HIV-1 envelope from codon-optimized gene sequences. *Current HIV Research* 2004;3(2):191-8.
- [157] Bower JF, Yang X, Sodroski J, Ross TM. Elicitation of Neutralizing Antibodies with DNA Vaccines Expressing Soluble Stabilized Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Trimers Conjugated to C3d. *J Virol* 2004 May 1, 2004;78(9):4710-9.
- [158] Pancera M, Lebowitz J, Schon A, Zhu P, Freire E, Kwong PD, et al. Soluble Mimetics of Human Immunodeficiency Virus Type 1 Viral Spikes Produced by Replacement of the Native Trimerization Domain with a Heterologous Trimerization Motif: Characterization and Ligand Binding Analysis. *J Virol* 2005 August 1, 2005;79(15):9954-69.
- [159] Pancera M, Wyatt R. Selective recognition of oligomeric HIV-1 primary isolate envelope glycoproteins by potentially neutralizing ligands requires efficient precursor cleavage. *Virology* 2005;332(1):145-56.
- [160] Sanders RW, Vesanen M, Schuelke N, Master A, Schiffner L, Kalyanaraman R, et al. Stabilization of the Soluble, Cleaved, Trimeric Form of the Envelope Glycoprotein Complex of Human Immunodeficiency Virus Type 1. *J Virol* 2002 September 1, 2002;76(17):8875-89.
- [161] Yang X, Farzan M, Wyatt R, Sodroski J. Characterization of Stable, Soluble Trimers Containing Complete Ectodomains of Human Immunodeficiency Virus Type 1 Envelope Glycoproteins. *J Virol* 2000 June 15, 2000;74(12):5716-25.
- [162] Yang X, Lee J, Mahony EM, Kwong PD, Wyatt R, Sodroski J. Highly Stable Trimers Formed by Human Immunodeficiency Virus Type 1 Envelope Glycoproteins Fused with the Trimeric Motif of T4 Bacteriophage Fibrin. *J Virol* 2002 May 1, 2002;76(9):4634-42.
- [163] Hammonds J, Chen X, Fouts T, DeVico A, Montefiori D, Spearman P. Induction of Neutralizing Antibodies against Human Immunodeficiency Virus Type 1 Primary Isolates by Gag-Env Pseudovirion Immunization. *J Virol* 2005 December 1, 2005;79(23):14804-14.
- [164] Barnett SW, Lu S, Srivastava I, Cherpelis S, Gettie A, Blanchard J, et al. The Ability of an Oligomeric Human Immunodeficiency Virus Type 1 (HIV-1) Envelope Antigen To Elicit Neutralizing Antibodies against Primary HIV-1 Isolates Is Improved following Partial Deletion of the Second Hypervariable Region. *J Virol* 2001 June 15, 2001;75(12):5526-40.
- [165] VanCott TC, Mascola JR, Kaminski RW, Kalyanaraman V, Hallberg PL, Burnett PR, et al. Antibodies with specificity to native gp120 and neutralization activity against primary human immunodeficiency virus type 1 isolates elicited by immunization with oligomeric gp160. *J Virol* 1997 June 1, 1997;71(6):4319-30.
- [166] Yang X, Wyatt R, Sodroski J. Improved Elicitation of Neutralizing Antibodies against Primary Human Immunodeficiency Viruses by Soluble Stabilized Envelope Glycoprotein Trimers. *J Virol* 2001 February 1, 2001;75(3):1165-71.
- [167] Srivastava IK, Stamatatos L, Legg H, Kan E, Fong A, Coates SR, et al. Purification and Characterization of Oligomeric Envelope Glycoprotein from a Primary R5 Subtype B Human Immunodeficiency Virus. *J Virol* 2002 March 15, 2002;76(6):2835-47.
- [168] Zhang CWH, Chishti Y, Hussey RE, Reinherz EL. Expression, Purification, and Characterization of Recombinant HIV gp140. *Journal of Biological Chemistry* 2001 October 26, 2001;276(43):39577-85.
- [169] Schulke N, Vesanen MS, Sanders RW, Zhu P, Lu M, Anselma DJ, et al. Oligomeric and Conformational Properties of a Proteolytically Mature, Disulfide-Stabilized Human Immunodeficiency Virus Type 1 gp140 Envelope Glycoprotein. *J Virol* 2002 August 1, 2002;76(15):7760-76.

- [170] Si Z, Phan N, Kiprilov E, Sodroski J. Effects of HIV type 1 envelope glycoprotein proteolytic processing on antigenicity. *AIDS Res Hum Retroviruses* 2003;19(3):217-26.
- [171] Adams O, Scheid A. Stepwise Deletion of the HIV Type 1 Glycoprotein 41 N Terminus Leads to an Increasing Export of Microvesicles Containing Uncleaved Env Glycoprotein. *AIDS Research and Human Retroviruses* 2001;17(14):1345-56.
- [172] McCune JM, Rabin LB, Feinberg MB, Lieberman M, Kosek JC, Reyes GR, et al. Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. *Cell* 1988;53(1):55-67.
- [173] Green TD, Montefiori DC, Ross TM. Enhancement of Antibodies to the Human Immunodeficiency Virus Type 1 Envelope by Using the Molecular Adjuvant C3d. *J Virol* 2003 February 1, 2003;77(3):2046-55.
- [174] Huang Y, Kong W-p, Nabel GJ. Human Immunodeficiency Virus Type 1-Specific Immunity after Genetic Immunization Is Enhanced by Modification of Gag and Pol Expression. *J Virol* 2001 May 15, 2001;75(10):4947-51.
- [175] Gallichan WS, Woolstencroft RN, Guarasci T, McCluskie MJ, Davis HL, Rosenthal KL. Intranasal Immunization with CpG Oligodeoxynucleotides as an Adjuvant Dramatically Increases IgA and Protection Against Herpes Simplex Virus-2 in the Genital Tract. *J Immunol* 2001 March 1, 2001;166(5):3451-7.
- [176] Horner AA, Ronaghy A, Cheng P-M, Nguyen M-D, Cho HJ, Broide D, et al. Immunostimulatory DNA Is a Potent Mucosal Adjuvant. *Cellular Immunology* 1998;190(1):77-82.
- [177] Kang S-M, Compans RW. Enhancement of Mucosal Immunization with Virus-Like Particles of Simian Immunodeficiency Virus. *J Virol* 2003 March 15, 2003;77(6):3615-23.
- [178] McCluskie MJ, Davis HL. Cutting Edge: CpG DNA Is a Potent Enhancer of Systemic and Mucosal Immune Responses Against Hepatitis B Surface Antigen with Intranasal Administration to Mice. *J Immunol* 1998 November 1, 1998;161(9):4463-6.
- [179] Moldoveanu Z, Vzorov AN, Huang WQ, Mestecky J, Compans RW. Induction of immune responses to SIV antigens by mucosally administered vaccines. *AIDS Res Hum Retroviruses* 1999;15(16):1469-76.
- [180] Ball JM, Henry NL, Montelaro RC, Newman MJ. A versatile synthetic peptide-based ELISA for identifying antibody epitopes. *Journal of Immunological Methods* 1994;171(1):37-44.
- [181] Cole K, Paliotti M, Murphey-Corb M, Montelaro R. Maturation of envelope-specific antibody responses to linear determinants in monkeys inoculated with attenuated SIV. *J Med Primatol* 2000;29((3-4)):220-30.
- [182] Miller MA, Murphey-Corb M, Montelaro RC. Identification of broadly reactive continuous antigenic determinants of simian immunodeficiency virus glycoproteins. *AIDS Res Hum Retroviruses* 1992;8(6):1153-64.
- [183] Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, et al. Human Immunodeficiency Virus Type 1 env Clones from Acute and Early Subtype B Infections for Standardized Assessments of Vaccine-Elicited Neutralizing Antibodies. *J Virol* 2005 August 15, 2005;79(16):10108-25.
- [184] Montefiori D. *Current Protocols in Immunology*. UNIT 1211 Evaluating Neutralizing Antibodies Against HIV, SIV, and SHIV in Luciferase Reporter Gene Assays: John Wiley & Sons, Inc, 2004.
- [185] Chertova E, Bess JW, Jr., Crise BJ, Sowder II RC, Schaden TM, Hilburn JM, et al. Envelope Glycoprotein Incorporation, Not Shedding of Surface Envelope Glycoprotein

- (gp120/SU), Is the Primary Determinant of SU Content of Purified Human Immunodeficiency Virus Type 1 and Simian Immunodeficiency Virus. *J Virol* 2002 June 1, 2002;76(11):5315-25.
- [186] Dettenhofer M, Yu X-F. Highly Purified Human Immunodeficiency Virus Type 1 Reveals a Virtual Absence of Vif in Virions. *J Virol* 1999 February 1, 1999;73(2):1460-7.
- [187] Mouland AJ, Mercier J, Luo M, Bernier L, DesGroseillers L, Cohen EA. The Double-Stranded RNA-Binding Protein Staufen Is Incorporated in Human Immunodeficiency Virus Type 1: Evidence for a Role in Genomic RNA Encapsidation. *J Virol* 2000 June 15, 2000;74(12):5441-51.
- [188] Tang S, Murakami T, Cheng N, Steven AC, Freed EO, Levin JG. Human Immunodeficiency Virus Type 1 N-Terminal Capsid Mutants Containing Cores with Abnormally High Levels of Capsid Protein and Virtually No Reverse Transcriptase. *J Virol* 2003 December 1, 2003;77(23):12592-602.
- [189] Wang C-T, Lai H-Y, Li J-J. Analysis of Minimal Human Immunodeficiency Virus Type 1 gag Coding Sequences Capable of Virus-Like Particle Assembly and Release. *J Virol* 1998 October 1, 1998;72(10):7950-9.
- [190] Doan LX, Min L, Changyi C, Qizhi Y. Virus-like particles as HIV-1 vaccines. *Reviews in Medical Virology* 2005;15(2):75-88.
- [191] Young KR, Ross TM. Particle-based Vaccines for HIV-1 Infection. *Current Drug Targets - Infectious Disorders* 2003;3:151-69.
- [192] Smith J, Amara R, McClure H, Patel M, S. S HY, Chennareddi L, et al. Multiprotein HIV-1 clade B DNA/MVA vaccine: construction, safety, and immunogenicity. *AIDS Res Hum Retroviruses* 2003;20(6):654-65.
- [193] Chan DC, Fass D, Berger JM, Kim PS. Core Structure of gp41 from the HIV Envelope Glycoprotein. *Cell* 1997;89(2):263-73.
- [194] Hill CP, Worthylake D, Bancroft DP, Christensen AM, Sundquist WI. Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. *Proceedings of the National Academy of Sciences of the United States of America* 1996;93(7):14804-14.
- [195] Tan K, Liu J, Wang J, Shen S, Lu M. Atomic structure of a thermostable subdomain of HIV-1 gp41. *Proceedings of the National Academy of Sciences of the United States of America* 1997;94(23):12303-8.
- [196] Center RJ, Schuck P, Leapman RD, Arthur LO, Earl PL, Moss B, et al. Oligomeric structure of virion-associated and soluble forms of the simian immunodeficiency virus envelope protein in the prefusion activated conformation. *Proceedings of the National Academy of Sciences of the United States of America* 2001 December 18, 2001;98(26):14877-82.
- [197] Grundner C, Yuxing Li, Mark Louder, John Mascola, Xinzhen Yang, Joseph Sodroski and Richard Wyatt. Analysis of the neutralizing antibody response elicited in rabbits by repeated inoculation with trimeric HIV-1 envelope glycoproteins. *Virology* 2005 5 January 2005;331(1):33-46.
- [198] Rao M, Matyas GR, Vancott TC, Birx DL, Alving CR. Immunostimulatory CpG motifs induce CTL responses to HIV type I oligomeric gp140 envelope protein. *Immunol Cell Biol* 2004;82(5):523-30.
- [199] Bower JF, Sanders KL, Ross TM. C3d enhances immune responses using low doses of DNA expressing the HIV-1 envelope from codon-optimized gene sequences. *Current HIV Research* 2005;3(2):191-8.

- [200] Spenlehauer C, Saragosti S, Fleury HJA, Kirn A, Aubertin A-M, Moog C. Study of the V3 Loop as a Target Epitope for Antibodies Involved in the Neutralization of Primary Isolates versus T-Cell-Line-Adapted Strains of Human Immunodeficiency Virus Type 1. *J Virol* 1998 December 1, 1998;72(12):9855-64.
- [201] York J, Follis KE, Trahey M, Nyambi PN, Zolla-Pazner S, Nunberg JH. Antibody Binding and Neutralization of Primary and T-Cell Line-Adapted Isolates of Human Immunodeficiency Virus Type 1. *J Virol* 2001 March 15, 2001;75(6):2741-52.
- [202] Bou-Habib DC, Roderiquez G, Oravec T, Berman PW, Lusso P, Norcross MA. Cryptic nature of envelope V3 region epitopes protects primary monocyctotropic human immunodeficiency virus type 1 from antibody neutralization. *J Virol* 1994 September 1, 1994;68(9):6006-13.
- [203] Stamatatos L, Cheng-Mayer C. Structural modulations of the envelope gp120 glycoprotein of human immunodeficiency virus type 1 upon oligomerization and differential V3 loop epitope exposure of isolates displaying distinct tropism upon virion-soluble receptor binding. *J Virol* 1995 October 1, 1995;69(10):6191-8.
- [204] Burton DR, Desrosiers RC, Doms RW, Koff WC, Kwong PD, Moore JP, et al. HIV vaccine design and the neutralizing antibody problem. *Nature Immunology* 2004;5(3):233-6.
- [205] Fung MS, Sun CR, Gordon WL, Liou RS, Chang TW, Sun WN, et al. Identification and characterization of a neutralization site within the second variable region of human immunodeficiency virus type 1 gp120. *J Virol* 1992 February 1, 1992;66(2):848-56.
- [206] Gorny MK, Moore JP, Conley AJ, Karwowska S, Sodroski J, Williams C, et al. Human anti-V2 monoclonal antibody that neutralizes primary but not laboratory isolates of human immunodeficiency virus type 1. *J Virol* 1994 December 1, 1994;68(12):8312-20.
- [207] Putney SD, Matthews TJ, Robey WG, Lynn DL, Robert-Guroff M, Mueller WT, et al. HTLV-III/LAV-neutralizing antibodies to an E. coli-produced fragment of the virus envelope. *Science* 1986;234(4782):1392-6.
- [208] Calarese DA, Scanlan CN, Zwick MB, Deechongkit S, Mimura Y, Kunert R, et al. Antibody Domain Exchange Is an Immunological Solution to Carbohydrate Cluster Recognition. *Science* 2003 June 27, 2003;300(5628):2065-71.
- [209] Gorny MK, VanCott TC, Hioe C, Israel ZR, Michael NL, Conley AJ, et al. Human monoclonal antibodies to the V3 loop of HIV-1 with intra- and interclade cross-reactivity. *J Immunol* 1997 November 15, 1997;159(10):5114-22.
- [210] Gorny MK, Xu JY, Gianakakos V, Karwowska S, Williams C, Sheppard HW, et al. Production of site-selected neutralizing human monoclonal antibodies against the third variable domain of the human immunodeficiency virus type 1 envelope glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America* 1991;88(8):3238-42.
- [211] Gorny MK, Xu JY, Karwowska S, Buchbinder A, Zolla-Pazner S. Repertoire of neutralizing human monoclonal antibodies specific for the V3 domain of HIV-1 gp120. *J Immunol* 1993 January 15, 1993;150(2):635-43.
- [212] Trkola A, Purtscher M, Muster T, Ballaun C, Buchacher A, Sullivan N, et al. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *J Virol* 1996 February 1, 1996;70(2):1100-8.
- [213] Kennedy RC, Henkel RD, Pauletti D, Allan JS, Lee TH, Essex M, et al. Antiserum to a synthetic peptide recognizes the HTLV-III envelope glycoprotein. *Science* 1986;231(4745):1556-9.

- [214] Cleveland SM, Buratti E, Jones TD, North P, Baralle F, McLain L, et al. Immunogenic and Antigenic Dominance of a Nonneutralizing Epitope over a Highly Conserved Neutralizing Epitope in the gp41 Envelope Glycoprotein of Human Immunodeficiency Virus Type 1: Its Deletion Leads to a Strong Neutralizing Response. *Virology* 2000;266(1):66-78.
- [215] Cleveland SM, McLain L, Cheung L, Jones TD, Hollier M, Dimmock NJ. A region of the C-terminal tail of the gp41 envelope glycoprotein of human immunodeficiency virus type 1 contains a neutralizing epitope: evidence for its exposure on the surface of the virion. *J Gen Virol* 2003 March 1, 2003;84(3):591-602.
- [216] Modrow S, Hahn BH, Shaw GM, Gallo RC, Wong-Staal F, Wolf H. Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. *J Virol* 1987 February 1, 1987;61(2):570-8.
- [217] Reading SA, Heap CJ, Dimmock NJ. A novel monoclonal antibody specific to the C-terminal tail of the gp41 envelope transmembrane protein of human immunodeficiency virus type 1 that preferentially neutralizes virus after it has attached to the target cell and inhibits the production of infectious progeny. *Virology* 2003;315(2):362-72.
- [218] Hollier MJ, Dimmock NJ. The C-terminal tail of the gp41 transmembrane envelope glycoprotein of HIV-1 clades A, B, C, and D may exist in two conformations: an analysis of sequence, structure, and function. *Virology* 2005;337(2):284-96.
- [219] Tochikubo K, Isaka M, Yasuda Y, Kozuka S, Matano K, Miura Y, et al. Recombinant cholera toxin B subunit acts as an adjuvant for the mucosal and systemic responses of mice to mucosally co-administered bovine serum albumin. *Vaccine* 1998;16(2-3):150-5.
- [220] Tilney NL. Patterns of lymphatic drainage in the adult laboratory rat. *J Anat* 1971;109(Pt. 3):369-83.
- [221] Sminia T, Draal G. Nasal-associated lymphoid tissue. In: Ogra PL, Lamm ME, Bienenstock J, Mestecky J, Stober W, McGhee JR, editors. *Mucosal Immunology*. London: Academic Press, 1999: 357-64.
- [222] Thomson MM, Perez-Alvarez L, Najera R. Molecular epidemiology of HIV-1 genetic forms and its significance for vaccine development and therapy. *The Lancet Infectious Diseases* 2002;2(8):461.
- [223] Berman PW, Gregory TJ, Riddle L, Nakamura GR, Champe MA, Porter JP, et al. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature* 1990;345(6276):622-5.
- [224] Cho MW, Kim YB, Lee MK, Gupta KC, Ross W, Plishka R, et al. Polyvalent Envelope Glycoprotein Vaccine Elicits a Broader Neutralizing Antibody Response but Is Unable To Provide Sterilizing Protection against Heterologous Simian/Human Immunodeficiency Virus Infection in Pigtailed Macaques. *J Virol* 2001 March 1, 2001;75(5):2224-34.
- [225] Johnston MI, Flores J. Progress in HIV vaccine development. *Current Opinion in Pharmacology* 2001;1(5):504-10.
- [226] Polacino P, Stallard V, Klaniecki JE, Montefiori DC, Langlois AJ, Richardson BA, et al. Limited Breadth of the Protective Immunity Elicited by Simian Immunodeficiency Virus SIVmne gp160 Vaccines in a Combination Immunization Regimen. *J Virol* 1999 January 1, 1999;73(1):618-30.
- [227] Lemiale FB, Denys ; Lebigot, Sarah ; Verrier, Bernard ; Buzelay, Laurence ; Brunet, Sylvie ; Barin, Francis. Immunogenicity of Recombinant Envelope Glycoproteins Derived From T-Cell Line-Adapted Isolates or Primary HIV Isolates: A Comparative Study Using Multivalent

- Vaccine Approaches. JAIDS Journal of Acquired Immune Deficiency Syndromes 2001 15 april 2001;26(5):413-22.
- [228] Slobod KS, Lockey TD, Howlett N, Srinivas RV, Rencher SD, Freiden PJ, et al. Subcutaneous Administration of a Recombinant Vaccinia Virus Vaccine Expressing Multiple Envelopes of HIV-1. European Journal of Clinical Microbiology & Infectious Diseases 2004;23(2):106.
- [229] Lu S. Immunogenicity of monovalent and polyvalent DNA prime/protein boost formulation encoding primary HIV-1 Env antigens as tested in rabbits and non-human primate models. Keystone Symposia. Keystone, CO, 2004.
- [230] McBurney SP, Ross TM. Developing Broadly Reactive HIV-1/AIDS Vaccines: A Review of Polyvalent and Centralized HIV-1 Vaccines. Current Pharmaceutical Design 2007;13:1957-64.
- [231] Feng Gao H-XL, Beatrice H. Hahn, Norman L. Letvin, Bette T. Korber, and Barton F. Haynes. Centralized HIV-1 Envelope Immunogens and Neutralizing antibodies. Current HIV Research 2007.
- [232] Gao F. Consensus and Ancestral State of HIV Vaccines. Science 2003;299:1515-8.
- [233] Gao F, Weaer, E.A., Lu, Z. Antigenicity and Immunogenicity of a synthetic HIV-1 Group M consensus envelope glycoprotein. Submitted 2004.
- [234] Kulken C. HIV-1 consensus sequences. HIV-1 Sequence Compendium 2002.
- [235] Nyambi P. Multivariate analysis of HIV-1 neutralization data. J Virol 1996;70:6235-43.
- [236] Gaschen B. DIversity considerations in HIV-1 vaccine selection. Science 2002;296:2354-60.
- [237] Green TD, Montefiori DC, Ross TM. Enhancement of antibodies to the human immunodeficiency virus type 1 envelope by using the molecular adjuvant C3d. J Virol 2003 Feb;77(3):2046-55.
- [238] Young KR, Smith JM, Ross TM. Characterization of a DNA vaccine expressing a human immunodeficiency virus-like particle. Virology 2004;327(2):262-72.
- [239] Lowe DM, Parmar V, Kemp SD, Larder BA. Mutational analysis of two conserved sequence motifs in HIV-1 reverse transcriptase. FEBS Lett 1991 May 6;282(2):231-4.
- [240] Mizuno A, Ido E, Goto T, Kuwata T, Nakai M, Hayami M. Mutational analysis of two zinc finger motifs in HIV type 1 nucleocapsid proteins: effects on proteolytic processing of Gag precursors and particle formation. AIDS Res Hum Retroviruses 1996 Jun 10;12(9):793-800.
- [241] Le Grice SF, Naas T, Wohlgensinger B, Schatz O. Subunit-selective mutagenesis indicates minimal polymerase activity in heterodimer-associated p51 HIV-1 reverse transcriptase. Embo J 1991 Dec;10(12):3905-11.
- [242] Tilney NL. Patterns of lymphatic drainage in the adult laboratory rat. J Anat 1971;109:369-83.
- [243] Huang Y, Kong WP, Nabel GJ. Human immunodeficiency virus type 1-specific immunity after genetic immunization is enhanced by modification of Gag and Pol expression. J Virol 2001 May;75(10):4947-51.
- [244] Gallichan WS, Woolstencroft RN, Guarasci T, McCluskie MJ, Davis HL, Rosenthal KL. Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract. J Immunol 2001 Mar 1;166(5):3451-7.
- [245] Kang SM, Compans RW. Enhancement of mucosal immunization with virus-like particles of simian immunodeficiency virus. J Virol 2003 Mar;77(6):3615-23.

- [246] Horner AA, Ronaghy A, Cheng PM, Nguyen MD, Cho HJ, Broide D, et al. Immunostimulatory DNA is a potent mucosal adjuvant. *Cell Immunol* 1998 Nov 25;190(1):77-82.
- [247] McCluskie MJ, Davis HL. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *J Immunol* 1998 Nov 1;161(9):4463-6.
- [248] Moldoveanu Z, Vzorov AN, Huang WQ, Mestecky J, Compans RW. Induction of immune responses to SIV antigens by mucosally administered vaccines. *AIDS Res Hum Retroviruses* 1999 Nov 1;15(16):1469-76.
- [249] Bower JF, Yang X, Sodroski J, Ross TM. Elicitation of neutralizing antibodies with DNA vaccines expressing soluble stabilized human immunodeficiency virus type 1 envelope glycoprotein trimers conjugated to C3d. *J Virol* 2004;78:4710-9.
- [250] Bower JF, Sanders KL, Ross TM. C3d enhances immune responses using low doses of DNA expressing the HIV-1 envelope from codon-optimized gene sequences. *Curr HIV Res* 2005 Apr;3(2):191-8.
- [251] Montefiori DC. Evaluating neutralizing antibodies against HIV, SIV and SHIV in a luciferase reporter gene assay. In: Coligan JE, Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., Strober, W., and Coico, R., editor. *Current Protocols in Immunology*: John Wiley & Sons, 2004.
- [252] Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, et al. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. *J Virol* 2005 Aug;79(16):10108-25.
- [253] Li M, Salazar-Gonzalez JF, Derdeyn CA, Morris L, Williamson C, Robinson JE, et al. Genetic and Neutralization Properties of Subtype C Human Immunodeficiency Virus Type 1 Molecular env Clones from Acute and Early Heterosexually Acquired Infections in Southern Africa. *J Virol* 2006 December 1, 2006;80(23):11776-90.
- [254] Roederer M, Koup RA. Optimized determination of T cell epitope responses. *Journal of Immunological Methods* 2003;274(1-2):221-8.
- [255] McBurney SP, Young KR, Ross TM. Membrane embedded HIV-1 envelope on the surface of a virus-like particle elicits broader immune responses than soluble envelopes. *Virology* 2007;358(2):334-46.
- [256] Crooks ET, Moore PL, Franti M, Cayan CS, Zhu P, Jiang P, et al. A comparative immunogenicity study of HIV-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120. *Virology* 2007;366(2):245-62.
- [257] UNAIDS. AIDS epidemic update: December 2006: Joint United Nations Programme on HIV/AIDS and World Health Organization; 2006 December 2006.
- [258] Brandtzaeg PB, E.S.; Farstad, I.N.; Jahnsen, F.; et al. Regional specialization in the mucosal immune system: what happens in the microcompartments? *Immunology Today* 1999;20:141-51.
- [259] Kaneko HB, I.; Wierzbicki, A.; et al. Oral DNA vaccination promotes mucosal and systemic immune responses to HIV envelope glycoprotein. *Virology* 2000;267:8-16.
- [260] McGhee JRM, J. In defense of mucosal surfaces: development of novel vaccines for IgA responses protective at the portals of entry of microbial pathogens. *Infect Dis Clin N Am* 1990;4:315-41.

- [261] Verthelyi D, Wang, V.W., Lifson, J.D., Klinman, D.M. CpG oligodeoxynucleotides improve the response to hepatitis B immunization in healthy and SIV-infected rhesus macaques. *AIDS* 2004;18:1003-8.
- [262] Verthelyi D, Kenney RT, Seder RA, Gam AA, Friedag B, Klinman DM. CpG oligodeoxynucleotides as vaccine adjuvants in primates. *J Immunol* 2002 Feb 15;168(4):1659-63.
- [263] Verthelyi D, Klinman DM. Immunoregulatory activity of CpG oligonucleotides in humans and nonhuman primates. *Clin Immunol* 2003 Oct;109(1):64-71.
- [264] Leifer CA, Verthelyi, D., Klinman, D.M. Heterogeneity in the human response to immunostimulatory CpG oligodeoxynucleotides. *J Immunother* 2003;26:313-9.
- [265] McBurney SP, Young KR, Ross TM. HIV-1 Envelope on the Surface of a Lentiviral Particle Elicits Broader Immune Responses than Soluble Envelopes. *Journal of Virology* 2005:submitted.
- [266] Yao Q, Vuong V, Li M, Compans RW. Intranasal immunization with SIV virus-like particles (VLPs) elicits systemic and mucosal immunity. *Vaccine* 2002 Jun 7;20(19-20):2537-45.
- [267] Wu HY, Nguyen HH, Russell MW. Nasal lymphoid tissue (NALT) as a mucosal immune inductive site. *Scandinavian Journal of Immunology* 1997;46(5):506-13.
- [268] Sminia TD, G. Nasal-associated lymphoid tissue. In: Ogra PLL, M.E.; Bienenstock, J.; Mestecky, J.; Stober, W.; McGhee, J.R., editor. *Mucosal Immunology*. London: Academic Press, 1999: 357-64.
- [269] Johansson E-L, Wassen L, Holmgren J, Jertborn M, Rudin A. Nasal and Vaginal Vaccinations Have Differential Effects on Antibody Responses in Vaginal and Cervical Secretions in Humans. *Infect Immun* 2001 December 1, 2001;69(12):7481-6.
- [270] Kozlowski PA, Cu-Uvin S, Neutra MR, Flanigan TP. Comparison of the oral, rectal, and vaginal immunization routes for induction of antibodies in rectal and genital tract secretions of women. *Infect Immun* 1997 April 1, 1997;65(4):1387-94.
- [271] Nardelli-Haeffliger D, Wirthner D, Schiller JT, Lowy DR, Hildesheim A, Ponci F, et al. Specific Antibody Levels at the Cervix During the Menstrual Cycle of Women Vaccinated With Human Papillomavirus 16 Virus-Like Particles. *J Natl Cancer Inst* 2003 August 6, 2003;95(15):1128-37.
- [272] UNAIDS. 2008 UNAIDS Annual Report: Joint United Nations Programme on HIV/AIDS; 2009.
- [273] Robertson DL, J.P. Anderson, J.A. Bradac, et al. A Reference Guide to HIV-1 Classification. In: Kuiken CL, B Foley, B.H. Hanh, et al., editor. *Human retroviruses and AIDS: A Compilation and Analysis of Nucleic and Amino Acid Sequences*. Los Alamos, NM: Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, 2000: 492-505.
- [274] Barnett SWa, Srivastava IKa, Kan Ea, Zhou Fa, Goodsell Aa, Cristillo ADb, et al. Protection of macaques against vaginal SHIV challenge by systemic or mucosal and systemic vaccinations with HIV-envelope. *AIDS* 2008;22(3):339-48.
- [275] Bogers WMJM, Davis D, Baak I, Kan E, Hofman S, Sun Y, et al. Systemic neutralizing antibodies induced by long interval mucosally primed systemically boosted immunization correlate with protection from mucosal SHIV challenge. *Virology* 2008;382(2):217-25.
- [276] McBurney SP, Ross TM. Viral sequence diversity: challenges for AIDS vaccine designs. *Expert Review of Vaccines* 2008;7(9):1405-17.

- [277] McBurney SP, Ross TM. Human immunodeficiency virus-like particles with consensus envelopes elicited broader cell-mediated peripheral and mucosal immune responses than polyvalent and monovalent Env vaccines. *Vaccine* 2009;27(32):4337-49.
- [278] Rollman E, Hinkula J, Arteaga J, Zuber B, Kjerrstrom A, Liu M, et al. Multi-subtype gp160 DNA immunization induces broadly neutralizing anti-HIV antibodies. *Gene Therapy* 2004 july 2004;11(14):1146-54.
- [279] Simon V, Ho DD, Abdool Karim Q. HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. *The Lancet* 2006 2006/8/11/;368(9534):489-504.
- [280] Verthelyi D, Kenney RT, Seder RA, Gam AA, Friedag B, Klinman DM. CpG Oligodeoxynucleotides as Vaccine Adjuvants in Primates. *J Immunol* 2002 February 15, 2002;168(4):1659-63.
- [281] Fernández-García A, M.T. Cuevas, M. Muñoz-Nieto, A. Ocampo, M. Pinilla, V. García, et al. Development of a Panel of Well-Characterized Human Immunodeficiency Virus Type 1 Isolates from Newly Diagnosed Patients Including Acute and Recent Infections. *AIDS Research and Human Retroviruses* 2009;25(1):93-102.
- [282] Harouse JM, Gettie A, Tan RCH, Blanchard J, Cheng-Mayer C. Distinct Pathogenic Sequela in Rhesus Macaques Infected with CCR5 or CXCR4 Utilizing SHIVs. *Science* 1999 April 30, 1999;284(5415):816-9.
- [283] O'Connor DH, Mothe BR, Weinfurter JT, Fuenger S, Rehauer WM, Jing P, et al. Major Histocompatibility Complex Class I Alleles Associated with Slow Simian Immunodeficiency Virus Disease Progression Bind Epitopes Recognized by Dominant Acute-Phase Cytotoxic-T-Lymphocyte Responses. *J Virol* 2003 August 15, 2003;77(16):9029-40.
- [284] Zhang Z-Q, Fu T-M, Casimiro DR, Davies M-E, Liang X, Schleif WA, et al. Mamu-A*01 Allele-Mediated Attenuation of Disease Progression in Simian-Human Immunodeficiency Virus Infection. *J Virol* 2002 December 15, 2002;76(24):12845-54.
- [285] Lore K, Larsson M. The role of dendritic cells in the pathogenesis of HIV-1 infection. *Apmis* 2003;111(7-8):776-88.
- [286] Wilflingseder D, Banki Z, Dierich MP, Stoiber H. Mechanisms promoting dendritic cell-mediated transmission of HIV. *Molecular Immunology* 2005;42(2):229-37.
- [287] Montefiori DC, Safrit JT, Lydy SL, Barry AP, Bilska M, Vo HTT, et al. Induction of Neutralizing Antibodies and Gag-Specific Cellular Immune Responses to an R5 Primary Isolate of Human Immunodeficiency Virus Type 1 in Rhesus Macaques. *J Virol* 2001 July 1, 2001;75(13):5879-90.
- [288] Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, et al. Vaccination with ALVAC and AIDSVAX to Prevent HIV-1 Infection in Thailand. *N Engl J Med* 2009 October 20, 2009;NEJMoa0908492.
- [289] Inglis S, Shaw A, Koenig S. Chapter 11: HPV vaccines: Commercial Research & Development. *Vaccine* 2006;24(Supplement 3):S99-S105.
- [290] Stanley M, Lowy DR, Frazer I. Chapter 12: Prophylactic HPV vaccines: Underlying mechanisms. *Vaccine* 2006;24(Supplement 3):S106-S13.